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under the vague and obscure term "Hant's cirrhosis". Many were due to congenital obliteration of the bile ducts and a few to portal or Laennec's cirrhosis and to toxic cirrhosis.

A study of the histopathology of the cases from Edinburgh along with those of 'Infantile Cirrhosis' of India is undertaken in order to determine if the latter disease is peculiar to India only and if it is not so to find out the possibility of a common aetiological factor responsible for these cases.

TABLE OF DISORDERS OF THE LIVER FROM THE POSTMORTEM RECORD OF THE ROYAL HOSPITAL FOR SICK CHILDREN, EDINBURGH, FROM JANUARY 1927 TO DECEMBER 1940

Year	P.M.	Cirrhosis of unknown aetiology	Cong. sph. cirrhosis	Cong. oblit. bile duct	Leuc. & sub-acute atrophy	Icterus gravis	Other disorders
1927	157	1	1		1	1	
1928	146	1	2	1	1		
1929	181					1	
1930	166		1				
1931	149	1	3			1	
1932	157					1	
1933	154	2	2				
1934	127						1 central necrosis
1935	159	1	1				1 cholecystitis
1936	149	1	1	1			
1937	193				1		1 central necrosis
1938	185	1	3			1	2 fatty livers
1939	150			1	3	1	
1940	175	1					
Total 14 yrs	2240	9	14	3	6	6	6

MATERIAL AND METHODS OF STUDY

The material for study consisted of sections from sixteen cases six from India and ten from the Royal Hospital for Sick Children, Edinburgh. Though the mortality rate for 'Infantile biliary cirrhosis' is great in Calcutta (700 deaths or more per year) and Madras, there is scarcity of post mortem material. Of the six cases available two were obtained from Pathology Department of the Vizagapatam Medical College through the kind permission of Prof Bhaskara Menon, and the other four were supplied by Prof Ramachandra Rao of the Madras Medical College. These sections were fixed in formalin and preserved in glycerine and water.

Of the ten cases from the Royal Hospital for Sick Children, Edinburgh, one was an authentic case of congenital obliteration of bile duct, and the other nine cases of cirrhosis of obscure etiology in children under two years of age. Paraffin blocks for eight of these cases and section of the liver and other organs stained with haematoxylin and eosin in the case of the remaining two were placed at my disposal by Dr MacGregor.

STAINING METHODS USED

- (1) For routine staining Meyer's acid alumina haematoxylin and eosin
- (2) For connective tissue Heidenhain's Azan method
- (3) Verheoff's elastic tissue stain
- (4) Sudan III counterstained with haematoxylin for fat
- (5) Dobell's modification of Levaditi for spirochaetes
- (6) Koppelleoff and Bermann's modification of Gram's method for bacteria
- (7) Foot and Menard's technique for reticulum

Six of the sections from India which were preserved in glycerine and water were washed in water for 48 hours and postfixed in Helly's fluid before embedding in paraffin.

MORBID ANATOMY AND CYTOLOGY OF THE LIVER

Case I—V.V. Female infant Aged 2 years

POST-MORTEM Morbid anatomy—Smooth capsular surface. Edges sharp. Uniform bile staining of the parenchyma on section. Sclerotic thickening of the larger portal spaces.

Histology—The parenchyma is permeated with bands of wavy fibrous tissue isolating the hepatic cords into groups of varying size. In places the collagen fibres have insinuated themselves in between individual liver cells giving the whole field the appearance of interstitial cirrhosis. The parenchymal cords are widely separated by the gaping sinusoids and many of them show degenerative changes and vacuolation. The nuclei are faintly stained, the cytoplasm granular and eosinophilic. The potential lumen in the hepatic cords is filled in places by a plug of inspissated bile. In sections from different parts of the liver the microscopical changes are slightly different, the islands of regenerated liver cells being larger and degeneration less pronounced. Here and there among the degenerated liver cells oval or rounded islands of parenchymal cells are found to be in various stages of necrosis. The healthy cells around such islands are compressed and stretched over them a condition suggesting an oedema of the involved cells prior to their disintegration. There is moderate infiltration with polymorphs and lymphocytes in the necrotic debris.

Bile Ducts Proliferation of biliary canaliculi varies in different sections. Numerous long and tortuous or short and tubular bile ducts are embedded in thick wide bands of cellular fibrous tissue. In other parts, the fibrous tissue bands are devoid of any proliferated bile ducts. The ducts in the portal tract are unaffected their lining epithelium being healthy and there is absence of any infiltration with inflammatory cells. A few of the proliferated bile ducts contain inspissated bile, showing a direct continuity with healthy liver cells.

Hepatic Vein Branches of the hepatic vein are scarcely to be seen in the section but wherever they are found they are seen to be surrounded by dense fibrous tissue and their walls are thick and sclerosed. A few of the larger branches show endophlebitis and infiltration of the intima with inflammatory cells. Hepatic terminals are scarce, their walls being involved in similar sclerosis with obliteration of the lumen. The parenchymal cells around such central veins have completely disappeared, being replaced by cellular fibrous tissue.

The Portal Tracts There is no marked increase of fibrous tissue in the Glisson's capsule. There is no periductal sclerosis. The hepatic artery and portal vein show no noticeable change. There is slight fibrosis of the periphery of the lobules in the region adjacent to the portal tract which tends to magnify the apparent width of the tract.

As the histopathology of infantile cirrhosis of India has been frequently described by various writers, it is considered



FIG 1—Liver of Case I. Massive necrosis of liver cells. The healthy cells are compressed and stretched out over the islands of necrosis. There is moderate infiltration with lymphocytes and polymorphs.
H and E $\times 80$

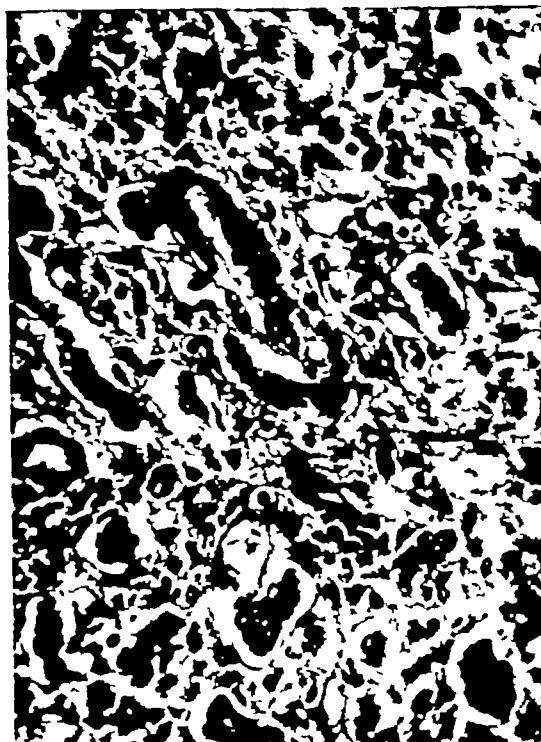


FIG 2—Liver of Case III. Numerous proliferated bile ducts are seen in the meshes of dense cellular fibrous tissue.
H and E $\times 300$



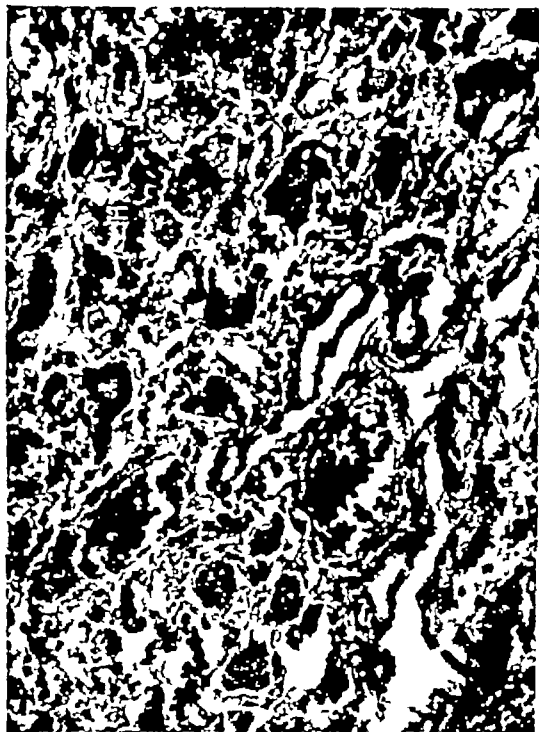


FIG 5—Liver of Case VIII Showing moderate proliferation of biliary canaliculi in a dense network of fibrous tissue. H—Islands of persistent haemopoiesis

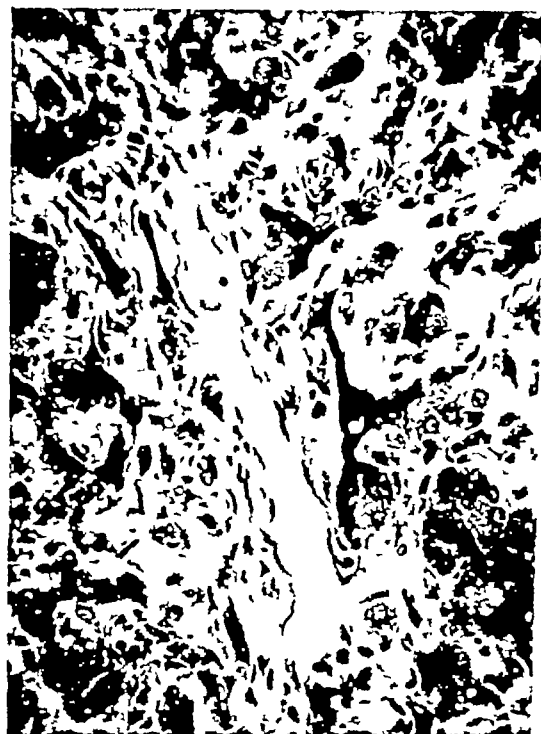


FIG 6—Liver of Case IX showing the longitudinal section of a central vein with marked thickening of its wall. The parenchyma around replaced by dense fibrous tissue hepatic cords and attenuated
H and E $\times 275$



FIG 7—Liver of Case X. Highly cellular fibrous tissue is seen to extend from the thickened wall of a central vein, and to permeate between hepatic cords. The picture is characteristic of interstitial cirrhosis
Azan $\times 300$

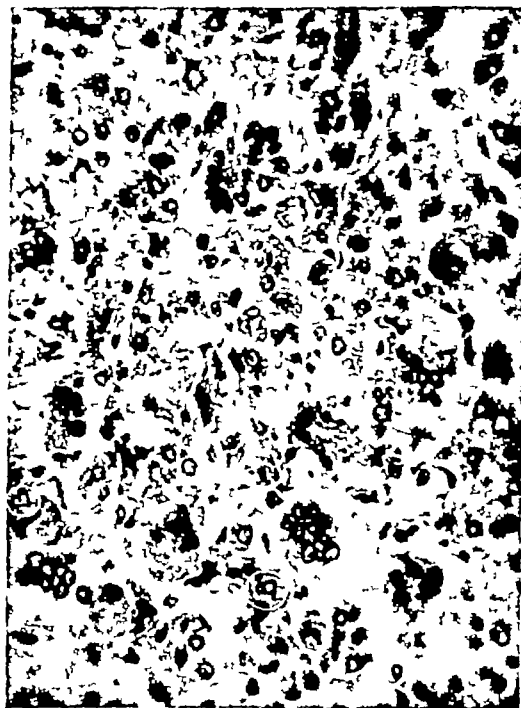


FIG 8—Liver of Case XVI with congenital obliteration of bile duct. Numerous multinucleated liver cells are seen. B. Inspissated bile in the lumen of the hepatic cords. N. Necrosis of parenchymal cells
H and E $\times 300$

that description of the histological findings in the other five cases of infantile biliary cirrhosis from India is not necessary.

Case 7—I.C., Male infant, aged 3 weeks

Liver. *Macroscopic appearance.*—Slightly enlarged, and firm when cut. Projecting from the surface were a number of nodules which corresponded in cut section with regeneration liver tissue. Islands of normal parenchyma were distributed irregularly in a deeply bile stained fibrous matrix. The gall bladder contained a small amount of bile and the ducts were patent.

Microscopic anatomy.—The normal parenchymal architecture is completely changed by bands of loose highly cellular fibrous tissue which encircles and isolates pseudolobules of regenerated parenchyma as well as small groups of hepatic cords and single cells. The cytoplasm of the parenchymal cells is granular, vacuolated and invariably shows the presence of bile pigment. Here and there masses of liver cells are undergoing necrosis, their cytoplasm disintegrating into granular bile-stained debris and their nuclei showing karyorrhexis and lysis resulting in the collapse of the sinusoidal reticulum. In these areas there is great proliferation of biliary canaliculi which vary in their character from structure closely resembling hepatic cords to those of fully developed biliary ducts. Plugs of inspissated bile are found in the biliary cords as well as in some of the newly formed bile canaliculi. The portal tracts do not show any definite increase in fibrous tissue or evidence of inflammatory cells.

The epithelium of the bile duct is intact and the lumen is patent. The branches of the hepatic vein show slight fibrosis of their walls and there is slight but apparent infiltration of them all by lymphocytes, endothelioid cells and very few polymorphs. Coarse granular pigment is observed in the

atrophy in a liver which shows evidence of slowly progressing toxic cirrhosis.

Case 8—J.W. Male infant aged 6 weeks

Macroscopic appearance.—Liver slightly smaller than normal. Colour deep green, surface finely granular and consistency firm. It was rather tough to cut and the cut surface suggested fine monolobular cirrhosis. Gall bladder normal. Cystic and common bile duct were patent.

Microscopic appearance.—The parenchyma is broken up into narrow tortuous columns and into small groups of cells by cellular and dense connective tissue. The normal lobular pattern is completely lost. Here and there small clumps of cells surrounding the thickened central vein have undergone necrosis and the transformation of the sinusoids into capillaries can be made out. The parenchymal cells are loaded with coarse and fine granules of greenish yellow bile pigment. Plugs of bile thrombi fill up the potential lumen in the hepatic cords. The outline of the cells is indistinct and the cytoplasm granular and vacuolated. Mitosis of cells and regeneration of lobules of liver cells are absent.

Portal tract. There is moderate increase in the fibrous tissue of the portal tract. The hepatic artery and portal vein are normal. The bile ducts are healthy, their lumen being patent and the lining epithelial cells intact and normal. There is some proliferation of the biliary canaliculi in the region adjacent to the portal tract and a few of them are distended with plugs of inspissated bile.

Kupffer cells show moderate proliferation. Many of them contain fine granules of bile pigment in their cytoplasm while in others the nuclei are elongated and spindle shaped and represented a gradual transformation of these cells into fibroblasts.

is deeply pigmented with bile, and their nuclei are irregular in shape and distribution and has affected single cells and isolated cords, but these patches of necrosis seem to bear a closer relationship to the hepatic venous tree and its terminals than to the portal tract.

Portal tracts show a slight increase of fibrous tissue of a cellular character. The hepatic artery and the portal vein are normal. There is a definite but not marked, proliferation of bile ducts. The lumen of the ducts are patent, and there is no inflammatory cell infiltration.

Hepatic venous tree shows a moderate thickening of its wall, and this change has affected the hepatic terminals (central veins) as well. Narrow bands of fibrous tissue can be seen to proceed from the central vein into the surrounding tissue. In the larger branches of the hepatic vein there is a slight round cell infiltration of the wall.

The Kupffer cells are immensely swollen, polymorphic and are stained greenish yellow with bile.

Case 12—MC Female infant, aged 10 months

Macroscopic appearance—The liver was smaller than average, surface nodular, and of a light yellowish brown colour. It was tough to cut and on the surface could be seen numerous firm nodules separated by fibrous tissue. Gall bladder and ducts were normal.

Microscopic appearance—Narrow bands of fibrous tissue extending from portal tract to portal tract has divided the parenchyma into islands of varying sizes. Extensive fatty change has affected most of the liver cells throughout the lobule. The cytoplasm and nuclei are well stained. There are no areas of necrosis. No hyaline change recorded by Mallory as being characteristic of portal cirrhosis can be seen in the neighbourhood of the Glissons' sheath.

Portal tract. There is a slight increase of fibrous tissue in the portal tract, and this extends beyond, the capsule to link up with the adjacent portal tracts. The resulting picture is that of a multilobular cirrhosis. The walls of the portal veins are relaxed and the lumen is distended with red cells. There is no sclerosis of the portal vein or hepatic artery. The bile ducts are patent and healthy. Proliferation of the bile ducts is slight. A few inflammatory cells can be observed in the portal tract and in the parenchyma abutting on it. Islands of regenerating liver cells are scarce, but a few such islands containing multi nucleated liver cells and cells showing mitosis are seen.

The sinusoids are collapsed and empty.

The Kupffer cells are impregnated with finely granular bile pigment.

Hepatic venous tree shows no thickening of the coats and there is no inflammatory cell infiltration.

Case 13—EM Female infant, aged 3 months

Morphology—The liver was greatly enlarged, light yellowish green in colour with a finely nodular surface. It had a firm consistency, was tough to cut having a sharp edge, and the cut surface suggested a monolobular cirrhosis. Gall bladder was healthy and contained bile. There was no obvious obstruction in the bile ducts.

Histology—Loose cellular fibrous tissue extending from the portal tract has encircled masses of liver cells, isolating them into lobules. There is some invasion of the lobules by fine strands of collagen fibres, but it is nowhere so extensive as to distort the parenchymal pattern. Most of the liver cells show marked fatty degeneration, a few are necrotic. Plugs of bile thrombi are seen in between the hepatic cords. A few of the liver cells and Kupffer cells are loaded with bile pigment.

Portal tract. There is moderate increase of fibrous tissue of a spongy and cellular type in the Glisson's capsule. It extends from each portal tract to the adjacent ones in narrow bands in which are numerous proliferated biliary canaliculi. Lymphocytic infiltration in these fibrous bands is slight. The artery, vein and duct are normal. There is no thickening or sclerosis of the coats of vessels. The lumen of the bile ducts is patent, but many of the newly-formed biliary canaliculi contain inspissated bile. The hepatic veins,

and the central veins do not show any abnormal changes. There is no phlebosclerosis nor endophlebitis.

Case 14—JN Female infant, aged 14 weeks

Macroscopic appearance—The liver was much enlarged, greenish yellow in colour, with a finely granular surface. Its consistency was firmer than normal, cut with resistance and the cut surface indicated a slight increase of fibrous tissue. A few nodules of yellow liver substance stood out clearly. Gall bladder was normal and contained bile. The ducts were patent.

Microscopic appearance—The parenchyma is oedematous with great distension of the lymph space of Disse, and collapse of the sinusoids. Many of the cells appear rather foamy and the differentiation between individual cells is more marked than normal. Most of the nuclei are well stained, but here and there some of them show karyorrhexis and lysis. An occasional bile thrombus is seen in some of the hepatic cords. A few widely distributed foci of haemopoiesis are revealed on close observation.

Glisson's Sheath. There is a slight increase of fibrous tissue of a loose and vascular character with scanty round cell infiltration. The arteries, veins and ducts appear normal.

The hepatic vein shows no definite change of the wall and the central veins are normal.

The notable feature of this case is the great distension of the lymph space of Disse, collapse of the sinusoid and the change of the reticulum of the sinusoids to collagen as observed with specific stain.

Case 15—AW Male infant, aged 3 weeks

Morphology—Size normal. Bright yellow in colour with red mottling. Appeared to be markedly fatty.

Histology—There is extreme fatty degeneration of the parenchymal cells. The architecture of the liver is well preserved. The fatty change has involved all the parenchymal cells and is of uniform distribution. No areas of necrosis or round cell infiltration are noted. The cytoplasm and nuclei are well stained. The portal tracts show a great increase in fibrous tissue. This increase is chiefly periductal, and follows the ramifications of the proliferated biliary canaliculi. A fair number of lymphocytes, polymorphonuclears, eosinophiles and plasma cells can be made out in the sclerosed periductal fibrous tissue, and in the cellular fibrous tissue following the track of the proliferated biliary afferents. The portal veins show no abnormal change, but some of the hepatic arteries show an adventitial thickening. There is a slight thickening of most of the hepatic terminals and a perarteritis with great narrowing of the lumen of hepatic arteries. There is just a suggestion of collagenous transformation of the sinusoidal reticulum fibres at the centre of some lobules. This is probably an early case of cholangitis lenta described by Naunyn and St. Klein, where there is diffuse pericholangitis but where the periductal sclerosis has not yet brought on any biliary stenosis or stagnation of bile.

Case 16—TP Male child, aged 8 months

Morphology—The liver slightly enlarged. Bile stained to a deep green colour. Surface coarsely granular. Section showed dense fibrosis in a close net work. Gall bladder was absent. Only a mass of fibrous tissue was seen lying at the posterior end of the gall bladder fossa. There was no common bile duct. The condition was congenital obliteration of the biliary passages.

Histology—The structure of the liver is not upset to any remarkable extent. The central veins are patent, and there is no necrosis at the centre of the lobules but numerous liver cells scattered throughout the lobule show icteric necrosis. Their cytoplasm is granular has taken the greenish yellow tinge of bile and their nuclei have disappeared. This wide-spread necrosis of isolated hepatic cells in the lobule has resulted in a certain amount of interstitial or intercellular fibrosis. There are numerous giant liver cells resembling foreign body giant cells with ten or more nuclei in them. No mitosis is seen. Wherever there is necrosis of cells polymorphonuclear leucocytes are much in evidence. Portal tracts

show a great increase of fibrous tissue, which has resulted in marked narrowing of the lumen of the portal vein. The absence of normal bile ducts in the Glisson's sheath in spite of the enormous increase of proliferated biliary canaliculi is noteworthy. Here and there bands of fibrous tissue bridge across adjacent portal tracts destroying the normal architecture of the parenchyma to some extent. The specific connective tissue reaction indicates the compact bundles of fibrous tissue irregularly disposed in the portal tract, from which delicate strands of connective tissue permeate into the neighbouring lobules along the course of the sinusoids. There is periarteritis and narrowing of the lumen of the hepatic artery.

No alteration is seen in the hepatic venous tree. There is no thickening of its branches, nor is there any increase of fibrous tissue in the adjacent region.

There is increase of biliary canaliculi in the portal tract. A few are branched and tortuous showing aneurysmal dilations plugged with inspissated bile. It is apparent that they are in direct communication with the hepatic cord which drains the bile into them but not with the bile duct.

The Kupffer cells are swollen and are packed with bile pigment.

The clinical and histopathological findings in these cases are shown in the table given below.

TABLE SHOWING A SUMMARY OF THE CLINICAL AND HISTOPATHOLOGICAL FINDINGS IN SIXTEEN CASES OF CIRRHOSIS OF THE LIVER IN INFANTS

Case No.	Age in months at autopsy	Family history	Diet	Clinical history	Type of cirrhosis
V A (I) I	24	Not available	Not available	Clay coloured stools, anaemia and oedema of lower extremities.	Toxic cirrhosis
A (I) II	36	Not available	Not available	Not available	Toxic cirrhosis with pericholangitis
P S (I) III	18	4th child to the mother. The 1st 3 had died of infantile biliary cirrhosis. Children by the 1st wife alive and well.	Mother's milk for 6 months supplemented with cow's milk from 3rd month and with rice from 12th month.	Not available	Toxic cirrhosis
P I (I) IV	7	10th child in the family. All the other children healthy.	Mother's milk for 6 months. Later supplemented with cow's milk.	Anaemia and leucocytosis. Culture of ascitic fluid sterile.	Toxic cirrhosis
P V (I) V	16	All the other children healthy.	Not available	Not available	Toxic cirrhosis
I (I) VI	27	Not available	Not available	Not available	Toxic cirrhosis
V C (I) VII	14	The younger sister died of the same disease later.	Cow's milk and sister's lactation.	Conjunctival icterus.	Toxic cirrhosis
V C (I) VIII	5	The elder brother died of the same disease.	Feasted for 9 weeks. Later supplemented with Airedale Lactogen and milk.	Charged from breast because not getting milk. Got first fully on change to	Acute liver atrophy superimposed on chronic passive congestion. Toxic

SYMPOSIUM ON ANTIBIOTICS

A Symposium on Antibiotics was held at the Scientific Section of XXIV All-India Medical Conference, Bombay on 28-12-47 with Dr A Erulkar as President

Speakers Dr B B YODH, Bombay—Antibiotics in General Medicine Dr RIBEIRO Bombay—Antibiotics in Skin Diseases Dr R V RAJAM, Madras—Antibiotics in Venereal Diseases

Antibiotics in General Medicine

B B YODH, MBBS MRCS, MRCP
Bombay

The meaning of this word 'antibiotic' is against living material, i.e. that which destroys living agents. It refers, however, to substances produced by or derived from living cells, which are capable of destroying other living cells. Even this meaning would include a large number of substances—not only penicillin, streptomycin, tyrocidine, gramicidin, bacteriophage, pyocyanase, chlorophyll, etc., but also other substances such as lysozyme and even antibodies as these are produced by living cells.

The term actually is used in a restricted sense to denote "antimicrobial" agents produced by living bacteria, yeasts, moulds and other plants.

The first fundamental fact about these agents is that the susceptibility of various organisms to them varies considerably and a proper knowledge of this well-worked out problem of susceptibility is essential. Penicillin, the most important of the group of antibiotics, acts against Gram-positive bacteria generally and destroys some of them as the streptococcus hemolyticus, some pneumococci and the gonococci easily, spirochaeta pallida, some leptospira, streptobacillus of rat bite fever fairly easily, while the staphylococcus and some streptococci with greater difficulty. The proper dosage and the frequency of administration can only be determined by a detailed knowledge of these susceptibilities.

From the point of view of proper treatment, therefore, isolation or a probable clinical guess of the infecting organism is the first necessary condition.

The next important fact is the quick absorption and equally quick excretion of the drug from the body. The drug acts only as long as it is in close contact with the infecting bacteria. If this contact disappears, the organisms, not killed, grow again and what is of greater importance especially with streptomycin, they become resistant to the action of the drug and grow in spite of its presence unless the concentration is markedly increased by a much larger dosage.

It has also been found that young growing forms are more easily attacked than older degenerated forms.

These facts indicate the paramount importance of keeping a constant level of concentration of the drug

in the blood throughout the course of treatment in all severe infections, and in all resistant strains of bacteria. Whenever the nature of infecting organism is not known and when it cannot be isolated, it would be wiser to give the drug in large doses and at short intervals.

It is permissible to rely on less frequent dosage even when it is large or incorporated in oil for slow absorption only when it is definitely known that the infecting organism is highly susceptible, is easily available for contact with the drug in the body, and the infection is comparatively mild and localised—as for example boils, small abscesses, localised cellulitis, etc.

This method of administration is not permissible if there is evidence of heavy septicæmic infection, infecting organism is unknown or when there is an inaccessible focus, such as osteomyelitis, infective endocarditis, cavernous sinus, thrombosis etc.

Considerable amount of work has been done on modes of administration. The best method of maintaining a continuous concentration is the continuous intravenous drip—the total 12 hourly dose being put in a saline flask at one time. Although there is the drawback of phlebitis at the site of venepuncture and the necessity of constant nursing care, it is the method of choice—at any rate for the first 24 to 48 hours in very heavy infections, and when organisms are not highly susceptible. After this period the mode of administration may be altered.

The next important method is the continuous intramuscular drip and this being somewhat painful one per cent novocaine 20 c.c. to a pint of solution may be added to relieve the pain. This method is also quite useful for the first few days in heavy infections.

The method of choice, however, is the intermittent intramuscular injection—although a large number of injections are required daily, 12 to 8 or 6 depending on the severity of the infection and sensitivity of the organism. This is slightly painful although well tolerated on the whole.

Majority of workers come back to this mode of administration after trying out various other methods as the most reliable in serious cases. For relief of pain one per cent novocaine ½ to 1 c.c. or local application of ice are useful.

Subcutaneous route is more painful and the absorption more erratic and hence not usually advised. In order to reduce the number of injections the oral route has been extensively experimented upon. The drug is absorbed from the gastro-intestinal tract, although the acid in the stomach probably destroys some of the drug. This is overcome by neutralisation by alkali or a protein buffer. The drawback, however, is that five times larger dose is required, and every two hours' or three hours' administration is essential. All workers are of the opinion that the oral route should be used only in mild cases and with extremely sensitive organisms, or in the latter part of the treatment of a severe infection to maintain concentration for a longer period.

Attempts at lowering the excretory rate have been made by a simultaneous administration of a competing chemical agent which will be excreted by the tubules and thus prevent the penicillin from being excreted. The sodium salt of para-amino-hippuric acid is used.

Benzoic acid which is converted by the liver into hippuric acid, orally 20 to 30 minutes before each intramuscular injection is recommended. This may increase the serum levels 4 to 8 fold.

Prolonging absorption is aimed at by injecting the drug dissolved in peanut oil and beeswax. It does so in the majority of cases but the absorption is often irregular and the method is uncertain in severe infections. It should be reserved for mild infections, when the infecting organism is highly sensitive or for prolonging the effect after the acute stage of a severe infection is overcome.

Local treatment—As the drug acts when brought in direct contact with the organism in infected tissues and even when purulent material is present, local treatment is extremely important. 20,000 to 40,000 units should be introduced in all infected cavities once a day after aspiration till the cavities clear up. This applies to all surface infections as well. Instillation or inhalation of the drug in the bronchial passages through the trachea has been eminently successful in pulmonary infections. Specially constructed aerosols have been used, and are attached to oxygen cylinders. One has used an adrenalin spray and connected one end with the tube from an oxygen cylinder, 50,000 to 100,000 unit dissolved in 1 cc being used each time.

Not only is there a good local action on the bronchial mucous membrane but a satisfactory general absorption as well. Intrathecal, intracisternal and even intraventricular administration are indicated in meningitis and cerebral infections when the organisms are sensitive to penicillin.

There are greater difficulties with Streptomycin administration than with penicillin. All the principles enunciated for penicillin apply to streptomycin except that as the excretion is slower 4 hourly administration may suffice. Resistant strains however develop far more frequently with Streptomycin if the concentration is not well maintained and that too for a long period. This is a real drawback in practice with the small quantities now available. The methods of administration are the same as those of penicillin, except that it is not yet used dissolved in peanut oil and beeswax, and is not given therefore, as a single daily dose.

Tyrothricin—A brief reference to the use of Tyrothricin locally (gramicidin and tyrocidine combined) is necessary. It is available as a cream and used as such or in suspension in distilled water 0.01 mg to 0.1 mg per cc. It is useful in infections by a variety of organisms not only however of the Gram-positive variety.

Gramicidin S or Soviet gramicidin is another substance discovered in an anaerobic sporulating bacillus of the *B. brevis* type. It is active against both Gram-positive and Gram-negative groups of organism and will be more useful than Tyrothricin when available.

There are still other antibiotics such as Streptothricin, Chlaram, Notatin, Penicillic acid etc. Chlorophyll is obtained from fresh or dried leaves by extraction with acetone. It is relatively non-toxic by parenteral administration to human beings. It possesses antibacterial activities *in vitro* against many organisms. *In vivo* it is not so strong. But topical applications are valuable in infected wounds, indolent ulcers, bed sores and also in cavities that are infected. More requires to be still known.

Antibiotics in Skin Diseases

REBELLO

Bombay

From time immemorial, it has been known that some living organisms destroy others or inhibit their growth. Pasteur and Joubert were the first to suggest that this phenomenon of antibiosis might be of use in the treatment of certain infections. The first attempt to apply an antibiotic for the purpose of treatment was made by Emmerich and Loew in 1899. Fleming's discovery has popularised antibiotics in medical therapeutics. The advent of penicillin and its use in staphylococcal and streptococcal infections opened a new field in dermatologic therapeutics. Though antibiotics are now very largely used on patients suffering from skin diseases, we are beginning to realise that antibiotics in dermatologic practice have a very limited use and that, mainly in pyodermas.

The usual antibiotics that have been tried in dermatological practice are penicillin and tyrothricin. Patulin obtained from *penicillium patulum*, has been tried by me in an ointment base in diseases due to fungi and the results have not been encouraging.

Streptomycin, produced by a strain of *Actinomyces griseus*, was described by Schatz, Bugie and Waksman in January, 1944. Owing to its limited availability it has not been extensively tried. I have practically no personal experience of streptomycin having used it in only one patient suffering from lupus erythematosus disseminatus, with unsatisfactory results, possibly due to insufficient dosage. In February last O'Leary and others reported the results of a few cases of cutaneous tuberculosis treated by them. They gave the patients one gram per day of a streptomycin salt in an aqueous solution intramuscularly in divided doses. They gave one patient two grams per day but the dose was discontinued because the patient complained of nausea, lassitude and general debility. The total dose ranged from 15 to 128 grams. They treated four cases of scrofuloderma, one of tuberculosis cutis colliquativa, one of tuberculosis miliaris disseminata faciei and two of lupus vulgaris. Those who had scrofuloderma and received the larger doses of streptomycin derived the most benefit. The results were encouraging but not very satisfactory. They considered the treatment inadequate in some of the cases. Their conclusions are that streptomycin although not the ideal agent for the treatment of cutaneous tuberculosis and tuberculides, does offer considerable encouragement because of its therapeutic efficiency in guinea-pigs inoculated with tuberculosis and because of the varying degrees of improvement in human beings. External and topical uses of streptomycin have not received much attention due to the difficulty in getting the antibiotic in sufficient quantities. There are reports that externally applied, streptomycin proved more valuable than older remedies in certain cases. It has been tried successfully in otitis externa and other infections due to *Pseudomonas aeruginosa* or in skin infections caused by colon bacilli.

Dubos in 1939 discovered an alcohol-soluble but water-insoluble substance called Gramicidin obtained from autolysed cultures of an aerobic, motile, spore-

forming soil bacillus called *Bacillus brevis*. Gramicidin is a high active agent against various Gram-positive organisms *in vitro*. Investigations on the chemical nature and purification of this antibiotic agent by Hotchkiss and Dubos showed that it contained a second substance known as Tyrocidine possessing some degree of activity against Gram-negative microorganisms. These investigators proposed the name Tyrothricin for designating the mixture of these two substances. Tyrothricin being too toxic for parenteral use in human beings, is employed only by topical application in the treatment of localised infections. It is supplied by the Mulford Laboratories of Sharpe and Dohme for topical application as a concentrate in alcoholic solution requiring dilution with sterile distilled water, which gives a cloudy preparation of the compound in colloidal suspension since it is not soluble in water. Saline solution should not be used as it produces a precipitation of the compound and prevents its even distribution in the diluting medium. The usual concentration for topical applications vary from 10 to 50 mgm per cent. Higher concentrations produce local irritation with pain or discomfort, hyperaemia, and cedema. Tyrothricin is more rapidly active than penicillin or sulphonamides. It has a low sensitizing potential. Tyrothricin has no systemic use, being too toxic for internal administration and hence any sensitization from local use will not interfere if any systemic therapy later becomes necessary. Moreover tyrothricin is stable and its solutions retain long their usefulness. The only apparent drawback is its systemic toxicity. Therefore care should be taken against too great and rapid absorption as is done when using mercurials or phenols as topical applications. Tyrothricin may be useful in certain cases of acne vulgaris, folliculitis, sweat-gland abscesses, infected cysts, other pyodermas and chronic infected ulcers. Sulzberger and another, report that tyrothricin-containing creams and ointments have proved ineffective in their hands.

Among the antibiotics, penicillin is the one that is most largely used in medical therapeutics. As I have said before its use in dermatologic practice is limited, being used chiefly in pyodermas. It is useful in infections due to penicillin-sensitive organisms or when a secondary infection with these organisms complicates the original condition. The majority of skin diseases which yield to penicillin are those due to *staphylococcus aureus* and *streptococcus pyogenes*. It has been found valuable in impetigo vulgaris, impetigo bullosa neonatorum and adultorum, ecthyma, infectious eczematoid dermatitis, burns and wounds, Bockhart's impetigo, sycosis vulgaris, furunculosis, carbuncle, some cases of multiple erythemas, erysipelas and other streptococcal infections, lymphangitis, lymphadenitis, acne conglobata et cystica, some cases of dermatitis herpetiformis, erysipeloid, in Vincent's angina, anthrax, syphilis and yaws. It has not been found of any significant value in acne vulgaris, atopic dermatoses, lichen planus, psoriasis, pemphigus vulgaris, all forms of sarcoidosis, all forms of tuberculoderms, in leprosy, lupus erythematosus, in diseases caused by fungi such as microspora, trichophyta, epidermophyta and the monilias, diseases caused by

filter-passing viruses and the coliform group. Some penicillin-insensitive organisms may even destroy penicillin so that in a mixed infection they indirectly protect penicillin sensitive organisms. Even in cases in which the organism is found sensitive to penicillin at first when treatment has got to be prolonged the organism may eventually develop a resistance to penicillin.

There are three methods of administration of penicillin in dermatologic practice — (i) as a topical application, (ii) parenterally (iii) orally.

TOPICAL USE

As a topical application penicillin may be used as a solution for spraying or for compresses or soaks. The solution is prepared by dissolving 100 to 1000 units of the sodium or calcium salt per millilitre of normal saline or distilled water. The calcium or sodium salt of penicillin may be incorporated in a cream or a water-soluble base or in an ointment in the strength of 100 to 1000 units of the salt to 1 gram of the base.

1. S. Jeffery's formula for a cream is as follows —

Arachis oil	125 ml
Lanette wax	60 gms
Water	275 ml

Heat the sterilized oil to 70 degrees centigrade add the wax. Heat the water to between 60 and 65 degrees centigrade. Add the water to the wax and oil with gentle stirring. Maintain a temperature of 65 degrees centigrade for 2 hours in order to sterilise. Store in 100 gm pots in a refrigerator until the cream is needed.

A formula for a water soluble base is as follows —

White Borden's ap powder	60 gms
Distilled water	100 ml
Total cream (cremule)	267 gm

When using the formula for the spray it should

The solution and the creams that are made with water have to be kept in a refrigerator to safeguard the activity of the penicillin. The stability of penicillin in aqueous solution is conditioned by temperature and pH. At pH 6.5 or when buffered at that pH solutions are at their maximum stability. Stock solutions should be stored in a refrigerator at 5 degrees centigrade when they may be reasonably expected to remain stable for one week without requiring re-standardizing. Certain antiseptics are useful adjuncts in aqueous preparations of penicillin to inhibit the development of micro-organisms insensitive to penicillin. Recommended for this purpose are chlorocresol 0.1 per cent phenoxetol and p-chlorophenol and chlorobutol.

The advantages of local application are — (i) A much higher concentration can be brought to the required site than is possible by systemic injections. (ii) There is great economy in penicillin. (iii) The treatment can be carried out by the patient himself.

The disadvantages of local application are — (i) The drug is useless if it cannot reach the organisms — because they are too deeply situated as in boils, carbuncles, anthrax and erysipelas. (ii) Occasionally the skin is irritated by either the penicillin itself or an impurity in it or in the constituent of the base in which it is applied. Solutions made in physiological saline irritate some skins due to the deposit of common salt.

Spraying should be done three or four times daily. Treatment by spraying is useful in hospitals, as a large number of patients can be treated without risk of cross infection. It is cheaper. There is no chance of irritation from the base as with a cream. The principal drawback is that the spray apparatus is expensive and tends to become choked and broken. Penicillin deteriorates more quickly in solution than it does in creams and ointments. Spray is not so convenient for individual use.

When prescribing an ointment to a patient the following instructions should be given:

1. Sterilise the skin before applying the ointment.

doses during the 24 hours. In this case the calcium salt of penicillin in an oil and beeswax base should be injected. The dose varies from 100,000 units to 300,000 units. For sometime now I have been treating patients with one or two large doses of the crystalline sodium salt of penicillin dissolved in normal saline. I have been employing doses ranging from 200,000 units to 500,000 units dissolved in 2 cc to 3 cc of normal saline.

ORAL USE

Orally penicillin-calcium tablets specially prepared for oral use may be tried when it is not possible to give the injections. The tablets contain either 25,000 units or 50,000 units. They may be given in doses of 1, 2 or more tablets at a time, every 2, 3, or 4 hours.

Before treating any dermatological condition with penicillin, the medical attendant should satisfy himself that the condition is produced by a penicillin-sensitive organism or the primary disease is complicated by a penicillin-sensitive organism. Laboratory control methods would be a useful guide. When it is not possible for one reason or another to have laboratory aid, the physician should be guided by his knowledge and experience. In treating superficial pyoderms like impetigo, ecthyma, infectious eczematoid dermatitis, Bockhart's impetigo, sycosis vulgaris or a superficial pyoderma as a complication of other dermatoses, one would resort to topical applications of penicillin. If the condition is acute, exudative and localised, penicillin compresses or soaks would be preferable. The compress should be changed three or four times. It should be covered with gutta serena or cellophane dressing. If the condition is acute, exudative and distributed in different parts of the body, it would be advisable to use a penicillin spray three or four times a day. In bullous impetigo the tops of bullae would have to be snipped and then the spraying done. In some cases the surface of the skin may be macerated or an oozing surface may be covered with scabs. The macerated skin or scabs should be removed and the skin cleansed with soaks of 1 in 9000 potassium permanganate solution each day before spraying or putting on the compresses. If the condition is sub-acute or chronic a cream or ointment would be preferable. Ambulatory patients prefer spraying in the day and an ointment at night. If there is no change at all in the condition in 5 or 6 days, it is better to stop the treatment. If the condition is better, the treatment should be continued for five days after involution in cases of simple pyoderma, upto two weeks in cases of slowly clearing or relapsing infections. Contact dermatitis sometimes occurs after four to five days of wet dressing or six to ten days of ointment therapy. This could be minimised by discontinuing the topical penicillin therapy for one or two days after four days' use of wet dressings or six days of application of ointment. During these two days I would recommend liniment calamine in an acute condition and a zinc paste in a chronic condition. If the eruption is very extensive or generalised, besides local treatment penicillin should be given parenterally and if

that is not possible, orally. In deep pyoderms like boils, hidradenitis, carbuncle, cellulitis, lymphangitis and lymphadenitis due to penicillin-sensitive organisms, penicillin should be given parenterally in doses ranging from 20,000 units to 50,000 units every three hours or in massive doses once or twice daily. The injections may be continued for 5 to 7 days or sometime more. In boils and hidradenitis, penicillin may be sprayed over the lesions and around as a preventive. I have treated a good number of cases of cellulitis, lymphangitis and boils with single massive doses of penicillin parenterally daily. In general the results have been very good. There have been relapses in cases of boils. When there are relapses it is imperative to find the cause and eliminate it. In the treatment of sycosis vulgaris of long-standing, you may be disappointed because of the relapses. Improvement of the general health of the patient is to be aimed at, no shaving of beard is to be allowed and clipping the hair instead would help the patient greatly.

REACTIONS TO PENICILLIN

I EPIDERMAL REACTIONS

(1) *Contact dermatitis*—Cohen and Pfaff have reported that 0.95 per cent of normal patients, who do not give a history of having used penicillin previously gave positive reactions to patch tests. According to the laws of dermatologic allergy this would indicate that a small percentage of the general public have been sensitized by some previous fungus infection and can be expected to react when penicillin is first applied to their skin. Penicillin in the strength usually applied in solutions or in ointments is not a primary cutaneous irritant. It should not in theory produce contact dermatitis when applied to the skin which has not been sensitized by previous applications of penicillin or by some other fungus. Pringle reports a 20 per cent incidence of dermatitis resulting from the local application of penicillin, particularly on the face. The percentage of dermatitis is considerably less when used in areas other than the face. It would be advisable to do patch tests before using the ointment over large areas.

(2) *Contact cheilitis, stomatitis and conjunctivitis*

II DERMAL TYPES OF REACTION

(1) The commonest is *urticaria*. Occasionally there may be severe urticaria associated with nausea, fever and painful, swollen joints as seen in serum sickness.

(2) *Angio-neurotic edema*

(3) *Toxic macular or scarlatiniform eruptions* may occur within the first few days after injections of penicillin.

(4) *Macules, papules and nodules of the erythema multiforme and nodosum type*

(5) *Phyloid type of reactions* may occur after injections of penicillin. I have seen this happen a few times, a few hours after the injection of penicillin. The distribution is usually on the hands and feet and the eruption is vesicular with severe pruritis.

(6) *Bullous eruptions* rarely occur

III COMBINED DERMAL AND EPIDERMAL REACTIONS have been observed by me

Sensitization may occur by topical application of penicillin or when taken orally or given parenterally. The most frequent reactions are urticaria and contact dermatitis. I have treated a good number of cases of syphilis and gonorrhoea with penicillin and only once have I seen a dermal reaction, in a case of gonorrhoea. Whilst in dermatologic cases, whether the penicillin was used topically, orally or parenterally at least 20 per cent of the patients showed skin reactions. Though penicillin used topically is a useful drug in superficial pyodermas some of these could be treated almost as efficiently with the older remedies without the risk of contact dermatitis and the higher cost the former entails. In deep pyodermas penicillin has a definite place.

In conclusion I have to state that the greatest and most common dermatologic therapeutic problems have not been solved by penicillin.

Antibiotics in Venereal Diseases

R. A. RAJAN, M.S., M.R.C.P.

Specialist in Venereal Diseases, Govt. General
Hospital, Madras

and

Professor of Venereal Diseases, Medical College,

mainly penicillin-G, which is most effective against organisms including *spirocheta pallida*. There is also some evidence that penicillin-X is more effective for gonorrhoea than either commercial penicillin or penicillin-G. In the meantime the research studies in America have been switched over to the evaluation of Crystalline Penicillin-G. It is quite possible in the future to have pure penicillin fractions like G and X for the treatment of syphilis and gonorrhoea separately and the evaluation studies with these pure fractions may upset or modify our existing knowledge about the value of penicillin in syphilis and gonorrhoea. At the present moment most of the evaluation studies are based on the use of commercial penicillin.

GONORRHOEA

The value of penicillin in gonococcal infections was promptly established after the clinical introduction of penicillin in 1943. Since that time, attempts are being made to determine the optimum dosage, the choice of the antibiotic, the method of administration, and the duration of treatment.

In early acute uncomplicated gonorrhoea or either sex, the following schedules have been reported to give a cure rate of more than 90 per cent and have the advantage of shortness of therapy, convenience, minimum discomfort to the patient and eminently suitable for treatment on an ambulatory basis.

In chronic, complicated, metastatic gonococcal infections and in gonococcal vulvo-vaginitis in children, the abbreviated schedules of therapy recommended for acute disease have been found to be unsatisfactory. A larger total dosage of 500,000 to 1 million units spread out over a longer period of time seems necessary in addition to certain adjuvant treatments like fever therapy and hormone therapy.

In chronic cases with the presence of other organisms, additional measures such as urethrovessicle irrigation, passage of bougies, prostatic massage, oral administration of one of the sulphonamides may be indicated. In the case of women, physical signs of discharge and irritation may persist even after penicillin therapy due to an associated infection with *trichomonas vaginalis*.

Recently clinicians from America and to a greater extent from Great Britain have been reporting an increasing number of cases of gonorrhoea resistant to penicillin therapy. In our experience, we have encountered penicillin resistant infections in quite a few cases. Is this the writing on the wall that is in the case of sulphonamides, the gonococcus is adapting itself to the antibiotic menace to its perpetuation? Time and further observation alone can tell.

In the meantime, laboratory and clinical studies of streptomycin in gonococcal infections reveal a promising additional weapon against the disease. In a few cases of penicillin-resistant gonorrhoea the administration of streptomycin in 3 to 5 gm total dosage have been reported to have cured the infections. We have no personal experience of this. Modern scientific medical research is providing one drug after another first the sulphonamides, then penicillin and thirdly streptomycin in the fight against the elusive gonococcus and one really wonders whether this strict human parasite, famous for its biological opportunism can ever be weeded out of the human population. The possibility of a coincident but potential syphilitic infection in patients treated for gonorrhoea with penicillin should always be borne in mind. Such patients should be watched for any clinical or serological evidence of syphilis for a minimum period of three months.

SYPHILIS

Penicillin therapy of Syphilis—1 In early syphilis the curative effect of penicillin therapy is directly proportional to the speed with which the diagnosis is made after the infection.

2 Best results are obtained in sero-negative primary syphilis with a reported cure rate of nearly 98 per cent.

3 In sero-positive early syphilis, including secondaries, the percentage of unsatisfactory results following penicillin therapy is still high (about 25 to 30 per cent). Hence it is suggested that in every case of sero-positive early syphilis, a combination of penicillin with other anti-syphilitic drugs, Mapharsen and bismuth should be given. There is evidence, both

experimental and clinical that a combination of penicillin with Mapharsen and bismuth or both, may give better results than penicillin alone. A synergistic action is postulated.

4 The recommended dosage of commercial sodium penicillin is 3.6 mega units for sero-negative primary syphilis and 5.4 mega units for sero-positive primary and secondary syphilis.

5 The incidence of the so-called reinfection or super-infection seems to be six to ten times more frequent following penicillin therapy than with the old standard metal chemotherapy.

Syphilis in Pregnancy—The most spectacular results have been reported in the prevention of prenatal syphilis following penicillin therapy in syphilitic pregnant women from the two University Clinics, Philadelphia and Baltimore. The prevention or cure rate of 98.4 per cent among the infants born is unequalled by any other form of therapy. In penicillin the results are equally good, no matter what the duration of pregnancy is at the time of treatment.

In a small number of eight cases of early infectious syphilis in pregnancy treated by us with penicillin, we were able to confirm the very favourable reports by the American workers. The risk of reaction to either the mother or the infant is almost negligible. The recommended dosage should not be less than 3.6 mega units and not more than 5.4 mega units administered intramuscularly, in aqueous or saline solution, third hourly, round the clock in single doses varying from 40,000 to 90,000 units. After treatment the mother should be followed up clinically and serologically, once a month till the time of delivery. After birth the infant must be followed for a minimum period of three months, clinically, serologically and roentgenographically.

Congenital Syphilis—Platou and co-workers from five co-operative university clinics in America, published an instructive study of the results of penicillin therapy in 252 infants with early congenital syphilis. They have reported 73 per cent satisfactory results, 9.1 per cent unsatisfactory results and 17.9 per cent in which the results are classified as uncertain. A mortality rate of 10.7 per cent from all causes is reported among the treated infants. In a group of 59 infants with early congenital syphilis, treated at the Venereal Clinic at Madras, 42 infants were discharged clinically cured with 5 deaths in the hospital. Of the 24 infants who have been followed and been under our observation from periods varying from one month to ten months, 20 are clinically well and 10 of them have been under our observation for more than four months and have achieved serological negativity. The following conclusions may be stated regarding the treatment of congenital syphilis with penicillin—

1 The recommended dosage of commercial penicillin is 50,000 units per pound of body weight to be administered in 120 equal divided doses during a period of 12 to 15 days.

2 In grossly debilitated marasmic infants the initial dose should be small of about 500 units gradually stepped up till a maximum of 5,000 units is reached in the course of a week or ten days.

3 Clinical response to the therapy has been uniformly satisfactory but it takes a longer time for the reversal of the serological reaction in early congenital syphilis than in early acquired syphilis.

4 Adequate and expert pediatric care is essential to successful penicillin therapy of congenitally diseased infants.

5 Most of the deaths reported during treatment occurred on the basis of malnutrition, dehydration and intercurrent infections.

In late congenital syphilis, particularly in interstitial keratitis the results of penicillin therapy are disappointing.

Neurosyphilis—Clinical and serological improvement have been reported in varying degrees in all types of neurosyphilis by the parenteral injection of penicillin in spite of the fact that penicillin does not penetrate the C.S.F. in any demonstrable quantity. In asymptomatic neurosyphilis and in acute syphilitic meningitis the results of penicillin therapy in doses of 3 to 5 mega units have been reported to be excellent.

In late primary optic atrophy, and syphilitic psychosis penicillin administered concurrently with and to other forms of therapy, has been reported to

in oil and wax has been reported by Thomas, Landy and Cooper of the Bellevue Hospital New York City. According to them, the treatment of early infectious syphilis with a single daily injection of 600,000 units of penicillin in oil and wax for eight days has proved very satisfactory. In a small series of six patients with early syphilis treated privately by me with a single daily injection of 300,000 units of penicillin in oil and wax for a period of 12 days and observed over a period of six to ten months, the results have been very satisfactory, clinically and serologically. The technical difficulty of administration, due to the high viscosity, the increased incidence of allergic effects and the cost of the drug will militate against its routine use in the treatment of syphilis. American workers Henry Welch and Romansky, are now collaborating on an extensive research to prepare penicillin in oil in a comparatively free flowing form and which will be as effective as the old viscid preparations. When the work is completed, liquid preparations of oil and wax may become available for the treatment of syphilis.

From studies on experimental syphilis, it is gathered, that streptomycin is very much less efficacious than penicillin and hence it is possible that the drug shall have no therapeutic future in syphilis.

In conclusion, it may be said that the treatment of syphilis with the antibiotics particularly penicillin is in a state of flux and four years is too short a period to assess its value in a disease which is as chronic, relapsing and subtle as syphilis.

DISCUSSIONS

DR P L DESHMUKH (*Poona*) said —Speakers before me have repeatedly voiced that penicillin has been grossly abused or misused. While agreeing with them in spirit I beg to sound a note of dissent on one point. Penicillin is relatively of recent introduction in therapeutics, and I believe that its potentialities are not yet completely explored. So, one can not be too dogmatic about its indications and contraindications except in a few cases. Laboratory research *in vitro* by itself is not sufficient to decide the fate of a remedy though it may throw a useful light on it. Clinical test alone is capable of giving the final verdict. So medical practitioners should be encouraged to try a new, safe and potent drug in different diseases and in tried diseases from a different point of view. Thus potentialities. For, in medicine, as we see in the alone it will be possible to reveal the drug in all its world around us, what may be considered as out of order to-day may prove to be the basis of order of the morrow.

This introduction may be considered as an apology for my further remarks. For, though the Plasmodium is known to be insensitive to penicillin, I tried the drug in cases of relapsing malaria with great success. This is how I came to use it in my first case. A lady was brought to me for repeated relapses of malarial fever though she had been liberally treated with injections of quinine, mepacrine tablets and quinine mixture, by her previous medical attendants. I thought of using penicillin in conjunction with the usual anti-malarials and wanted to see if relapses could be checked. I gave intramuscular injections of penicillin two lac units in 5 c.c. normal saline on three consecutive days simultaneously with injection of quinine hydrochlor gr 6 intramuscularly on the first and third days of penicillin. Mepacrine tablets were continued five days longer at one twice a day. To my surprise I found that the patient did not get a relapse to this day *i.e.*, during about a year. Further cases which I treated on the same line gave uniformly successful results clinically and bacteriologically. I believe the explanation of this success lies in the modern theory about the mechanism of a relapse. According to the modern conception, the Plasmodium is assumed to have two cycles in the human being *viz.*, a corpuscular and an extra-corpuscular cycle. The latter is also known as the 'reticulo-endothelial' or the 'X' cycle which has been definitely proved in birds. Relapses are explained on the assumption of overflow of parasites in the peripheral blood from the extra-corpuscular cycle. I assume that the usual anti-malarial drugs, which in reality form only the suppressive treatment of malaria, kill the parasites in the peripheral blood, while penicillin has a selective action on the extra-corpuscular cycle of the malarial parasite.

If my observations are corroborated by my colleagues here, a new and very useful vista will be opened for penicillin therapy.

DR G COELHO (*Bombay*) said Adequate facilities for investigation of a case are not available for the general practitioner, hence there is a danger for overprescribing.

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In pediatric use, injections into multiple boils, empyema cavities after aspiration of the pus is a useful procedure. The experience with typhoid fever treated with penicillin alone 50,000 units every 3 hours for 4 days is not conclusive because of the few cases, so treated, but I am inclined to think that it is beneficial in toxic cases. The clinical results in congenital syphilis treated with a dosage of 75,000 units per pound of weight distributed over 14 days, are superior to those obtained with arsenic, bismuth and mercury. Similar has been my experience with stomatitis. In diphtheria penicillin combined with antitoxin is valuable in toxic conditions, but in all cases of diphtheria the antitoxin should not be forgotten.

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DR BALDEV SINGH (*Amritsar*) said It is a recognized fact that the quantity of magnesium ribonucleic acid present in the organism determines the sensitivity of the organism to penicillin. In cases of typhoid fever and Malta fever if antiserum is given along with penicillin the diseases are easily vulnerable to the treatment. This is attributed to the fact that the corresponding organism is able to take a greater quantity of magnesium ribonucleic acid.

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DR P N PRADHAN (*Nagpur*) said State control of penicillin is necessary. Pharmacology of penicillin is a debatable point in regard to how it precisely acts. If it creates an unsuitable environment for the growth of the invading organism or if it acts on the growth factor, *viz.*, para-amino-benzoic acid or others. This point opens a wide field for the bacteriologist to study, why a particular organism is sensitive to penicillin. It is also important to determine how long it will take for the growth factor once it has been destroyed by penicillin to appear in circulating blood. Single massive dose of penicillin was found useful in a case of bilateral palmar abscess whereas in a case of rheumatic endocarditis, repeated courses of penicillin failed to be effective.

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DR M R CHOLKAR (*Nagpur*) said Penicillin is effective in breast abscess, with sinus as well as in unopened direct abscess. An article on a few cases of breast abscess treated in the way has appeared in an issue of *Lancet* of 1947.

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L R DESHPANDE (*Hyderabad, Dn*) said that he found good results with penicillin treatment in erythema nodosum, diphtheria, tetanus and typhoid.

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DR. C N SHROFF (*Bombay*) also spoke

BENEFITS OF OPEN AIR IN THE TREATMENT OF TUBERCULOSIS

TALWALKAR, M.B., (POM), F.P.C.S., (ENG),
Bombay

In India there are no special hospitals for tuberculosis of bones and joints. The average case is put in plaster of Paris or some sort of splint and treated at home or sent away to his native place. In all civilised countries the cases are treated in special hospitals far away from crowded cities and entirely in the open air. Foreign domination, poverty and ignorance have so far prevented such treatment from being undertaken in this country. The general surgeon is used to rapid operations and quick cures. He has not developed that patience which is necessary for prolonged, conservative treatment of these diseases. With increasing consciousness for the need of orthopaedic surgery, such hospitals however are bound to develop. They require specialists who can devote time and have the patience to look after the same patient for years, if necessary for very little remuneration.

It was Hugh Owen Thomas (1834-91) who recognised the value of open air treatment in tuberculosis of bone. He used to insist on his patients being kept outside in the open air, and it was a common sight in his day to see old men on their frames lying in the garden or on the street. His nephew, Sir Robert Jones (1858-1933) had the genius to appreciate what cured the cases. He found luckily in Miss Agnes Hunt, one of his patients, an admirable worker who organised a small hospital. From humble beginnings through the efforts of Sir Robert Jones and Miss

the plaster and the lying down. Mercer in 1946 in discussing prognosis of tuberculosis of bones and joints in his text book writes. Reference should be made to the great improvement in prognosis that has attended the efficient sanatorium regime now practically universal in this country. This shows the importance of open air in prognosis of tuberculosis of bones and joints. In these hospitals, besides the open air, there is a peculiar buoyant atmosphere. The psychological effect of seeing a fellow patient going out cured is a tonic which patients at home can never get. The sight of patients at various stages of improvement also helps a new patient considerably in bearing the tedium of lying down.

In an open air hospital these patients are kept in the verandah for twenty-four hours even on the coldest day. All are given both general and local rest of some sort in a plaster cast or a splint. They are given colloidal calcium with vitamin 'D' injection 2 cc. twice a week and a mixture containing iron and arsenic and glycerophosphates. Tuberculin in very minute doses (1/1000000) may also be given to these cases once a week, gradually increasing to 1/10000. The sedimentation rate is tested as a routine by Westergren's method and shows improvement with the clinical progress. Cod liver oil, calcium, vitamin D, ultra violet therapy are all adjuncts. But often they are used only as primary treatment.

The following cases which were treated in an open-air hospital may prove interesting.

REPORT OF CASES

Case 3—Master H, aged 11, admitted on 22-6-45 for bilateral tuberculosis of the ankle joints for seven years. He had been treated in various places in Sind and had been advised bilateral amputation of the leg. There were multiple sinuses on the left side and the ankle had swollen to three times its size. X-ray showed decalcification and the normal trabeculations had completely disappeared. The ankle on the right was not as bad as on the left and X-ray examination showed less destruction. His sedimentation rate was 100 mm per hour. The patient was having a maximum temperature of 101°F on admission. He was emaciated, anemic and looked very toxic. It seemed justifiable to amputate both the legs considering the toxicity and anaemia. He was however treated conservatively in the open air clinic and was put up in plaster of Paris upto above the knee. After one year he was looking better and X-ray examination showed that calcification was occurring. Eighteen months after this 2½ years after his admission the pale anaemic boy had become a dark, healthy adolescent. He had that peculiar dark complexion found in the Koli boys round Versova and Vasai. This change was due mainly to the open air near the sea-side. Recent X-ray films show that trabeculations have returned and calcification is almost normal. He will be discharged when the sinuses heal. The patient is now looking quite healthy and normal.

Case 4—C, female, aged 35, was treated for eighteen months in another hospital. She was admitted on 15-10-1945 with a sinus in the left groin and tuberculous lesion of the second and third lumbar vertebrae. Her temperature was 99.5°F and sedimentation rate was 85 mm per hour. The sinus healed in eight months and her temperature came to normal. Today she can walk about with a posterior support. Her sedimentation rate is now 20 mm per hour and X-ray examination shows satisfactory sclerosis.

Case 5—S G, female, aged 21, was admitted on 24-2-1946. On admission she had a swollen left knee joint with sinuses. It was tuberculous in nature and of eighteen months' duration. Her temperature was 100°F maximum. Her sedimentation rate was 80 mm per hour. The joint was immobilized in plaster of Paris till February, 1947. She had not improved very much during this time and sinuses still persisted. The temperature however had settled down to normal in three months' time. After February 1947, a Thomas' knee splint was given. The improvement since then has been remarkable and she has put on 20 lbs weight. In my opinion the Thomas splint immobilises the knee more satisfactorily than a plaster of Paris cast in tuberculous conditions. The muscles relax completely. In a plaster of Paris cast, the muscles can contract and often cause an abscess. Thomas, it may be mentioned here originally used his splint for tuberculosis of the knee. This patient would have required an amputation considering her anaemia, thin wasted appearance and sinuses. I feel that open air conservative treatment saved the limb.

Case 6—R G, male, aged 47, admitted on 11-12-1947, for tuberculosis of the right ankle joint with three sinuses. He had been advised amputation of the leg but had refused to get it done. On admission his sedimentation rate was 60 mm per hour. A crab splint and the usual line of treatment was adopted. The sinuses healed in two months and he improved rapidly. He has been discharged with a walking calipers with which he can walk about. His sedimentation rate is now 15 mm per hour. This patient but for the open air hospital, would have lost his leg.

CONCLUSION

I am personally convinced that the cases mentioned above would not have had such satisfactory results without the treatment in an open air hospital. In the open air hospital referred to above, 61 patients have been treated so far.

Following table shows the number of cases and the sites of tuberculous affection.

Site affected	Number of patients
Spine	40
Ankle	8
Hip	6
Knee	2
Sternum	2
Elbow	2
Metatarsal	1
Total	61

Out of the 40 cases of tuberculosis of the spine, 3 left the hospital prematurely, 1 because he could not afford further treatment, 1 due to leprosy and 1 because of language difficulty. 3 were cases in the terminal stage and they died. 1 early case also died from severe anaemia which could not be cured with liver extract injections, iron therapy and blood transfusions. 3 cases had tuberculosis of the lungs and were asked to go to some other hospital. Thus at least 31 patients out of these 40 cases of caries spine benefited by the open air treatment. It is however no good calculating the percentage of results on such a small number of cases. Still, it seems worth stressing that of the cases undergoing treatment when not in extremis, only 1 out of 31 died. This compares favourably with the mortality figures in Liverpool and Oxford (5 per cent immediate mortality).

In conclusion, may I reiterate that the object of this paper is to stress the value of open air hospitals for the treatment of bone and joint tuberculosis and the need for many such hospitals all over India.*

* Read at the XXIV All-India Medical Conference, Bombay, Dec. 1947.

SPECIAL ARTICLE

THE BRITISH PHARMACOPOEIA—1948

V ISWARIAH, B.A., M.B., M.R.C.P.,
Professor of Pharmacology, Andhra Medical College

The seventh revision of the British Pharmacopoeia has been published this year, the first having been published in 1864. The British Pharmacopoeia was expected to be revised at intervals of ten years, incorporating established new drugs and modifying, if not weeding out old and less serviceable ones. The last (sixth) was published in 1932. From then to now, no less than seven addenda were issued to take the place of the rightly due 1942 revision, in an attempt to keep up to date the 1942 revision, delayed due to war conditions.

EVOLUTION OF THE BRITISH PHARMACOPOEIA

It is interesting to study the evolution of pharmacopoeias in general and the British Pharmacopoeia in particular, as the study is just another aspect of progress of medicine.

The first work of its kind published under government authority appears to have been that of Nuremberg in 1543. A passing student, Valerius Cordus showed a collection of medical recipes which he had collected from the writings of eminent medical authorities, to the physicians of the town who impressed by it, urged him to print it for the benefit of the apothecaries. They obtained for his work sanction of the senatus. An earlier work known as "Antidotarium Florentinum" had been published under the authority of the college of medicine of Florence.

Until 1617, drugs and medicines as were in common use were sold in England by the apothecaries and grocers. A row between the grocers and the apothecaries resulted in the college of physicians in 1618 empowering only the apothecaries to dispense medicines and that according to specifications embodied in a Pharmacopoeia by the college of physicians. Thus the first London pharmacopoeia had included mostly the works of Mezerius and Nicolaus de Salerno. The compounds employed here were often heterogeneous mixtures of 20 to 70 ingredients. Among others the ingredients included oyster shell pearls crab eyes excrements of human beings dogs urine foxes calculi human skull and moss growing on it (probably the beginnings of penicillin) blind puppies cutworm etc. This pharmacopoeia existed till 1721 when under the auspices of Sir Hans Sloane important alterations were made deleting many of the useless and poisonous remedies. The tendency to simplify was carried to a much greater extent later and in 1788 the edition then published purged itself of the 1,000 compounds of 2,000 names old remedies. The 18th edition of the British Pharmacopoeia was published in 1864. The 19th edition was published in 1932. The 20th edition was published in 1948. The 21st edition was published in 1957 and the 22nd edition in 1963.

last in 1850. The preparations contained in the three united kingdom pharmacopoeias were not all of uniform strength and was naturally a source of much inconvenience and danger to the public. In consequence the medical act of 1858, ordained that the General Medical Council should publish a book containing a list of medicines and compounds to be called the British Pharmacopoeia for the whole of the British isles. Hitherto they were published in Latin and the first edition in English appeared in 1864.

A dissatisfaction was felt over this edition as the majority of the compilers were not engaged in the practice of pharmacy and the committee constituted as such was competent rather to decide upon the kind of preparation than upon the method of their manufacture (We hear today in India the echoes of the dissatisfaction, as India is striving to place pharmacy, drugs and pharmacopoeias in order). The necessity for this element in the construction of a pharmacopoeia is now fully recognised in every country, in most of which pharmaceutical chemists are represented on the committee for the preparation of the legally recognised manuals. National pharmacopoeias under the authority of government now exist in almost all advanced countries.

But the rapid increase in medical, pharmacological and pharmaceutical knowledge renders necessary frequent new editions. Each new edition of a pharmacopoeia requires several years of experimental work for developing suitable formulae and hence the production at intervals of such works as Squire's Companion to the Pharmacopoeia, Martindale's Extra Pharmacopoeia, New and Non-official remedies of the American Medical Association etc., that tend to keep the medicinal preparations abreast of time. With many medical practitioners, the Extra Pharmacopoeia is more the bible than the B.P. or the U.S.A. one. The British Pharmaceutical Codex is published under the authority of Pharmaceutical Society of Great Britain, in which the character and tests of purity of many non-official drugs, their preparations, glandular products, sera vaccines, etc., are given. This work may serve as a guide under the Foods and Drugs Act for the purity and strength of drugs not included in the B.P., and as a standard for the commercial grades of purity of those in the pharmacopoeia which are used for non-medicinal purposes as well.

INTERNATIONAL PHARMACOPOEIA

The desirability of a uniform formula for the more important medicaments is forced by the ever increasing facility for travel intercommunication and interrelation of nations in many spheres. Attempts have been made by international pharmaceutical and medical conferences to settle the basis on which an international pharmacopoeia could be prepared. But owing to the usual national jealousies and the attempt to include too many preparations it has been found that it is not possible to prepare a uniform pharmacopoeia. The British Pharmacopoeia is the only one which is still in use.

ting an Indian and a colonial addenda in 1900 made an attempt at unification in a limited sphere. The health organisation of the U N O, who knows, may attempt this.

INDIAN PHARMACOPOEIA

In 1900 an Indian and colonial addenda were permitted to the fourth revision of 1898. The Government of India edition of 1901 was accordingly published to suit "Indian conditions". Before the compilation of the 1932 edition, the civil research sub-committee on the British Pharmacopoeia suggested, "Where it is desired that official recognition should be given in any part of the empire to any local drugs or local substitutes we suggest that this should be left to the governments concerned, which by means of supplements or addenda to which they may accord the necessary sanctions, can meet any local requirements or introduce any modification or alteration desired". Accordingly an Indian pharmacopoeia list has been published in 1946. Its utility is yet to be established. It includes in the main several bitters, volatile oils, astringents for which Indian botany and materia medica are famed, malt, extract malt with shark liver oil, derris, pyrethrum, Paris green (insecticides) cobra and viper venoms, pilula rloes et ferri and rasfoetida (omitted by the new B P) etc.

THE BRITISH PHARMACOPOEIA OF 1948

The task of compiling the last edition has by no means been an easy one. The committee has been assisted by a clinical committee of 20 members, eight other committees, some of them with several sub-committees. The difficulty was enhanced by the fact that the compilers had to look not to the immediate future, but to 5 or 10 years ahead. It may happen that some preparation on the crest of its popularity finds no mention in the list while another which has virtually been discarded may find a place. The seven addenda from 1932 to 1945 had tended to bridge a wide gap which otherwise would have been felt and tended to keep the 1932 edition reasonably up-to-date war conditions and rapid therapeutic advances taken note of.

The main interest about the 1948 British pharmacopoeia (due in September this year) is the striking commentary it provides on the great therapeutic advances of the last decade or more. Ostensibly this is evidenced by the size of the new edition which has about 300 pages added to the previous one. About 150 entirely new monographs and about 155 new names have been introduced. It has incorporated new, none the less familiar preparations like 'Cremora', 'Tabella', 'Emulsio' etc. The official injections have been increased from 6 to 84.

As evidence also of its reorientation is the discarding of no less than 145 monographs of the 1932 B P.

Therapeutics, of late, having become a swiftly progressive art *cum* science, agents of questionable, under or objectionable utility have rightly been passed by. Old is not always gold, it sometimes tends to get mouldy and has to be deemed a sold out product. The exit of several drugs are for the reasons such as (1) their utility is not commensurate with their unwanted actions, (2) better substitutes, possibly cheaper are readily available and (3) occasionally one or other is not available or not possible of steady output. The new admissions of drugs are for precisely the reverse reasons *i.e.*, their established use based on known or recognised actions, their ready availability, etc.

In the inclusion of new medicaments, the new B P has maintained its traditional cautious conservatism and a considerable time-lag is noticeable between the introduction of new remedies and their final installation in the pharmacopoeia.

Though the Pharmacopoeial Committee was mainly concerned with the living down of standards of activity, its task of assessing clinical utility has been rendered easy by the labours of institutions like the 'Council of Pharmacy and Chemists' of the American Medical Association or the 'Therapeutic Research Council' of the British Medical Association that are more concerned with protecting the medical profession and the public against "fraud, undesirable secrecy, objectionable advertisements etc. in connection with the medicinal agents" and to assess drugs with reference "the status of medicinal articles it is importuned to use" by the manufacturer. The pharmacopoeia nevertheless should be the standard for a medical man who should seek its aid on all occasions. How many general practitioners own a pharmacopoeia? The pharmacopoeia also tends to enforce a medical discipline with regards nomenclature, dosage etc. Official names of proprietary preparations are not known to many. Sometimes one comes across a medical man who is shocked that the dispenser had violated his instructions in having given 'Sulphathiazole' when the doctor had prescribed 'Cibazol' as though a poison was substituted. Atebrin is better known than mepacrine and a good few are confused between mepacrine, pamaquin, plasmochin and quinacrine.

A legal difficulty of modern pharmacopoeia is the inclusion in some of them of synthetic chemical remedies, the process for preparing which have been patented and the substances are sold under the trade mark names such as cibazol. The scientific chemical name is often long and unwieldy and the physician by naming the drug by the patentee's puts the pharmacist or chemist in difficulties. If the chemist had no cibazol when prescribed, should he dispense thirzamide, in which case he lays himself open to attack by the patentee. The only plan therefore is for the physician to use the chemical official name which cannot be patented.

Instance comes to ones mind of a patient, a professor of economics, having a heated argument with the writer when cardophylline that was prescribed for

The old pituitary extract is trifurcated into injectio posterior pituitary, oxytocin and vasopressin. A new injection not familiar to many is injectio aethanolamine oleatis (0.9 per cent, 2 to 3 cc) used as a sclerosing agent in the treatment of varicose veins. Out of the 15 injections of 1932 B. P. five find an exit like injectio ferri injectio hydrargyri injectio sodii morrhuate etc. One fails to understand why injectio sodii chloridi et aceri of the 1932 B. P. has been replaced by Ringer solution or injectio sodii chloridi composita. Notable miscellaneous injections include those of diadine and iodine (for pyelography), summum sulphaphan, sodii thiosulphate sodii aurio, etc. (for myxomatosis) penicillin that has not established value in hard core penicillin had been called a "cure" for gonorrhea and gonorrhea prophylaxis.

preparations reasserting themselves despite urea stibamine and neostibosan. Altogether the 1948 B. P. list of injections numbering eighty and odd is very impressive. Parenteral medication as the route of choice gauged by efficiency, economy and energy seems to have gained recognition.

Tabella—Since the advent of the sulpha drugs and vitamin products in the form of tablets and the popularity of this form of medication with the public, tablets are recognised in the latest pharmacopoeia. The 1932 B. P. had just one tabella i. e., glycerilis trimitratris, though the 7th addendum included erythrol tetranitrate. The sublingual administration with evidence of better absorption here than in the stomach necessitated the inclusion of tablet form of the vasodilator. The non-recognition of tablets till now was due to the fact that when made by compression the tablets are often so hard and sometimes insoluble that they escape absorption and are often detected in the faeces. The specific methods prescribed for tablet making in the B. P. 1948 i. e. moist granulation or dry granulation or granulation by preliminary compression, seem to be intended to overcome the possible risk of nonabsorption.

There are 49 tabella in the new B. P. including the familiar sulpha compounds, various vitamins like ascorbic acid, nicotinic acid, acetaminophenone, aneurine hydrochloride etc., alkaloidal tablets like ephedrine, codeine, atropine, etc., several popular analgesics like aspirin, phenacetin, Dover's powder, etc. singly or in combination (some of the familiar proprietary ache tablets like *veganin*, *salidon*, etc., having secured recognition), hormones like thyroid, testosterone, progestin, a synthetic form of it named aethu-steronum hexoesterol, dienoestrol etc. It is interesting to note that of the sulpha compounds sulphathiazole, sulphadiazine, sulphacetamide, sulphaguanidine and succinyl sulphathiazole alone have gained recognition. Sulphapyridine, prontosil, pro-septasine etc., are passed by. If one may venture a forecast, sulphaguanidine, sulphacetamide and succinyl sulphathiazole may have a short lease of life, for the other three promise to be 'all comprehensive' between them.

The size of the tabella varies, the maximum size being half a gram. Dosage is in terms of the active principle always. The dosage of Sulphaguanidine is given as 0.5 to 2 Gm like those of sulphathiazole and sulphadiazine while the dose of succinyl sulphathiazole is given as 0.25 Gm per pound.

Cremora—These are just elegant versions of unguenta or pasta. The designation cream or cremora sounds less of a medicament and they are also looking more elegant, like to cosmetic creams. 500 units of sodium or calcium penicillin are to be in a gram of the cremora incorporated with hard and liquid paraffin, emulsifying wax and chlorocresol for mild sterilising. The preparation cremor penicillin sterilisatus without chlorocresol but sterilised by autoclaving and with more water is intended for injection intramuscularly. There

is also an unguentum penicillin. The two external medicaments of penicillin are yet to establish their utility.

Other new entrants—In the group of general anaesthetics are included aether vinicum or divinyl ether and trichlorethylene or trilene. The former is deemed of value in short operations particularly over the nasopharyngeal region, while the latter is finding an important place in the treatment of trigeminal neuralgia. Cyclopropane is already included in the 7th addendum.

The closely allied tetrachlorethylene has secured official recognition as a safe anthelmintic against hookworm.

Phenitoin sodium better known as dilantin sodium, a valuable anticonvulsant has become official. Amongst opium derivatives the notable change is the admission of papaverine (injection) as a valuable antispasmodic with a status equivalent to injectio theophylline ethylenediamine and the new pethidine hydrochloride (Pethidine is also analgesic). A significant change in the outlook is also noticed in the incorporation of codeine and even Dover's powder with aspirin as analgesics. Though so far one failed to notice in the innumerable patent analgesics like *aspro*, *salidon*, *veganin*, *novalgin*, *cibalgin* and *sandozol* etc., any inclusion of opium derivatives, the lead by the B. P. may tend to open the flood gates.

In the cardiac group of drugs introduction of tabella digitalis pulverata and tabella digoxin are no less noteworthy than the change in the nomenclature of strophanthin to ouabain. Infusion of digitalis is no more official. One is eagerly looking forward to the inclusion of digitoxin and the products with predominant vasoconstrictor sympathomimetic action. Substances claimed to have the latter action i. e., predominant peripheral vasoconstrictor action without the cardiac accelerating or cerebral action like *synephrin*, *propadrine*, *methedrine* etc., have not yet been recognised. Similarly on the parasympathetic side, atropine substitutes like *syntopan*, *neurotrasentin*, *eumydrin* etc., have not secured recognition except pethidine which has the added analgesic effect.

Thiouracil and methyl thiouracil are now included in the B. P. The list of vaccines have increased from 3 to 8 or more, including acne vaccine, cholera, pertussis, staphylococci, Flexner dysentericum, typhus and yellow fever vaccine. There are several modifications in the list of vaccines, sera and toxins that it is difficult to comment. The toxin list has included those of staphylococcus, and tuberculinum. The sera list has deleted antidysenteric and antipneumococcal sera.

It is hoped in concluding that the medical profession in the use of drugs would give heed to recognised medicaments as embodied in the B. P. and would also submit itself to the medical discipline in the choice, designation and dosage of therapeutic substances.

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RURAL MEDICAL RELIEF

Recently Rural Medical Relief is receiving much attention of the provincial and central governments. There is no doubt that the medical relief available in the rural areas is scanty and primitive. On the other hand in the periodic epidemics rural areas suffer most. It is a good augury that with the advent of freedom the question of rural medical relief is being viewed with a new perspective. Nearly 90 per cent of the people in India live in the rural areas and the basic problem before the country is the provision of adequate health protection to this large section of the community.

The Bhore Committee¹ discusses this problem in detail. It bases its argument and recommendation on the conception that the state should provide a complete health service as far as possible and include within its scope the largest possible proportion of the community. It also envisages that certain primary conditions essential for healthful living must in the first place be ensured. Suitable housing, sanitary surroundings, and a safe drinking water supply are pre-requisites of a healthy life. The Committee is of opinion that "under the conditions existing in the country, medical service should be free to all without distinction and that the contribution from those who can afford to pay should be through the channel of general and local taxation."

In all the plans of rural medical relief in the provinces, it is noticeable that no stress is being given on the pre-requisites of a healthy life. By that we do not mean that medical relief should be withheld till the pre-requisites are there. We have always held that everything must proceed according to a plan and no patch work should be started as a sop to the public demand.

It is a fact which government must face boldly that there are not enough doctors in the rural areas. Qualified doctors have always settled in towns for their living. If a doctor has got to live he must earn and there is no doubt that towns are better in this respect than villages. A professional man who has passed successfully through arduous and prolonged scientific education is not generally willing to reside in a remote country area where amenities of life are few and earnings meagre. The solution will be found in improved communication and increasing use of mechanical transport by the doctor who has initiative while the sick man will himself be able to travel to the nearest dispensary or hospital with less difficulty than is often imagined. We must say the problem is not been correctly approached by some provincial governments. Col. Amir Chand² in an illuminating article exposes the method of a certain provincial government regarding medical relief. The said government is considering the proposal whereby even a small number of medical students will be required to do a period of service in rural areas as a condition of their service in a rural area.

of one year before he or she would be granted a degree or diploma and that during the period of such service he or she will be granted a sum of Rs 50 per month as out of pocket expenses and the service will be considered as approved if it was certified by the Civil Surgeon of the district as such. We draw the attention of our readers to this article where Col. Amir Chand has shown that if this method is followed, it will help neither the rural population nor the doctor concerned and in his opinion such a move is dangerous and retrograde.

The efforts of another provincial government for rural medical relief are described in a communication to this Journal.³ We have no official communication denying this press report on which the letter is based. The provincial government have decided to start a medical school to which it is proposed to admit 56 students in the first instance. This school will have a 3 years course and a revised syllabus and students who have passed the matriculation examination will be eligible for admission to the school. It is understood that these 'doctors' will be allowed to practise in villages only and this move has been taken by the provincial government to meet the great shortage of medical practitioners in the rural areas.

One is amazed at the lack of a sympathetic attitude towards a villager. The argument seems to be that a halfbaked doctor is better than a quack. But this is a dangerous argument because it is difficult to prove who is the worst quack. We are forced to conclude that the government have never seriously considered the reasons why doctors do not go to settle in rural areas nor have they taken any steps to implement the Bhore Committee's report, even partially.

We only ask why this conscription and why this indecent haste to manufacture halfbaked doctors or quacks with official recognition? If it is a question of conscription it must also include all other professions as Col. Amir Chand forcefully argues in his article. Instead of making halfbaked doctors have the government seriously tried to attract the medical profession to the rural areas? Instances are not rare in other civilized countries where similar problems had to be faced. Did the government care to enquire how these problems were solved there? In the Highlands and Island service of the north of Scotland and in certain British colonies, the promising method has been to subsidize the practitioner or to provide him with transport or travelling allowance. Even in Soviet Russia there are special amenities like free house and free car, longer holidays with full pay etc⁴ for practitioners in sparsely populated areas.

The doctor must be given a living wage, the necessary amenities of life and facilities for education of his children before he can be attracted for a rural practice. Government should understand that a rural tented doctor will be more useful for the purpose for which he is being sent. The Indian Medical Association has offered its hand of co-operation to the government since August 1947. There is no reason why the government should not accept its offer and co-operate to solve this important and difficult problem.

NOTES AND NEWS

THE 3RD ANNUAL CONFERENCE OF GUJARAT, CUTCH AND KATHIAWAR

Under the auspices of the Gujarat and Kathiawar Provincial Branch, I M A, 3rd Annual Conference of the medical men of Gujarat, Cutch and Kathiawar will be held at Surat in March, 1949. The reception committee has been formed under the able chairmanship of Dr M K Dixit. Papers on important scientific subjects will be read and discussed by eminent medical men of the province. It is also decided to have an exhibition of surgical and medical appliances and pharmaceutical products. For further particulars please communicate to Dr K H Haradwalla, Organising Secretary, Vanki-Rordi, Surat.

ALL-INDIA DENTAL CONFERENCE

The Fourth All-India Dental Conference has been invited to hold its session in Calcutta on February 5, 6 and 7, 1949 by the Bengal branch of the All-India Dental Association.

The honorary organising Secretary of the Reception Committee announces that the Governor-General of India, H E Sri C Rajagopalachari has consented to be the patron of the conference.

STUDY OF DENTISTRY ABROAD

The following candidates have been selected on the Central Selection Board for advanced training abroad in dentistry under the Government of India scheme.

Central candidates 1 Mr Prem Prakash, 2 Mr Daman Lal, 3 Capt T Chudgar.

Provincial candidates 1 Dr T N Chawla (U P), 2 Dr Hari Dayal Gupta (U P), 3 Dr A N Khandadia (Bombay), 4 Dr Kashi Nath Paul (West Bengal).

States 1 Dr Brij Bahar Singh Marya, (Patiala), 2 Dr Ram Pratap (Nabha), 3 Dr Joginder Singh (Faridkot).

RESEARCH IN MENTAL DISEASES

Following the recommendation of the Bhore Committee, the Upgrading Committee appointed by the Government of India has selected the Mental Hospital, Bangalore, as a centre for research and post-graduate study in mental diseases. Dr M V Govindaswami, Superintendent of Bangalore Mental Hospital, will be the Director of post-graduate study and research.

LADY TATA MEMORIAL TRUST

The Trustees of the Lady Tata Memorial Trust announces the award of the following scholarships and grants for the year 1948-49.

International Awards for research in diseases of the blood with special reference to leucaemias.

1 Dr Jorgen Bichel, Denmark, 2 Dr Johannes Clemmensen, Denmark, 3 Dr C F M Plum, Denmark, 4 Dr Simon Iversen, Denmark, 5 Prof Edoard Storti, Italy, 6 Dr Pierre Cazal, France, 7 Dr Guido Totterman, Finland, 8 Dr Peter A Gorer, England, 9 Dr Edith Paterson, England, 10 Dr M C Bessis, France, 11 Dr A Kelemen, Hungary, 12 Dr & Mrs Paterson, Christie Hospital and Holt Radium Institute, Manchester, England.

Indian Scholarships of Rs 250 per month each for one year from 1st July 1948 for scientific investigations having a bearing on the alleviating of human suffering.

1 Mr Suprabhat Mukherjee, (Applied Chemistry), Calcutta, 2 Mr Yashwant Balkrishna Rangekar, (Biochemistry), Bombay, 3 Mr Gangagobinda Bhattacharya, (Diabetes), Calcutta, 4 Mr K Ramamurti (Biochemistry), Bangalore, 5 Miss V Shanta Iyenger, Bombay, 6 Mr B K Sur (Biochemistry), Bangalore.

CLINICAL TRIALS OF NEW REMEDIES UNDER THE I R F A

The Secretary, Governing Body and Scientific Advisory Board, in addressing all hospitals in India, Indian Research Fund Association, New Delhi, writes.

The Governing Body of the Indian Research Fund Association has on the advice of its Scientific Advisory Board, decided to establish under its auspices a 'Therapeutic Trials Committee' to sponsor and co-ordinate clinical research on new therapeutic agents. This Committee will encourage and aid impartial clinical trials of biological, chemotherapeutic and pharmaceutical agents of Indian or foreign origin, which offer promise in the prevention, treatment and diagnosis of diseases. The Medical Research Council of the United Kingdom and the Council of Pharmacy and Chemistry of the American Medical Association have set up similar bodies in their respective countries and it is considered that the time is now ripe for the initiation of such an organisation in India also. The Committee constituted by the Scientific Advisory Board of the I R F A for the purpose is as follows —

1 Dr M G Kini, late Superintendent, Stanley Hospital, Madras, 2 Dr B Mukerji, Director, Central Drugs Laboratory, Calcutta, 3 Lieut-Colonel Jaswant Singh, Director, Malaria Institute of India, Delhi, 4 Dr J C Patel, Honorary Assistant Physician, K. E. M. Hospital, Bombay, (Secretary).

This Committee is empowered to co-opt other members as and when required and its functions will be —

(i) to frame rules and regulations for the conduct of clinical trials,

(ii) to obtain necessary information regarding facilities available in different institutions in India for this type of work. This information will be obtained through the office of the Secretary, Indian Research Fund Association,

(iii) to decide in the first instance on the suitability of any particular drug for clinical trials, and

(iv) to collect data regarding suitability of the place and personnel who should be invited to undertake clinical trials of a particular type of drug.

The conditions under which the Indian Research Fund Association will be prepared to undertake clinical evaluation of new remedies of Indian or foreign origin submitted by the manufacturers have been drawn up on the analogy of those adopted by the Medical Research Council of the United Kingdom and a printed copy of this is enclosed for your information. To carry out satisfactorily clinical trials of such agents the Association will naturally require the co-operation and help of all medical institutions in India which have facilities for such work and with this end in view I write to request you kindly to let me know at your earliest convenience whether your Institution would be prepared to undertake clinical trials on behalf of the Indian Research Fund Association when asked to do so. It is requested in this connection that you would be so good as to fill in the attached questionnaire giving information under the various heads required by the Committee and send it to this office as soon as possible.

Facilities available in _____ for the
conduct of clinical trials

1 Name of Institution

2 Number of beds with the number of specialists and junior medical officers attached to each division

(a) Medical

(b) Surgical

(c) Gynaecology & Obstetrics

(d) Ophthalmology

(e) Chest Diseases

(f) Others

3 Is there a separate Children's hospital?

4 Is there a separate Dermatological ward?

5 Number of beds that can be set apart in each ward for controlled clinical study without hampering the routine work.

6 Does the Institution maintain an Out-Patients Department? If so, the average attendance (monthly or weekly) and the types of cases treated may be stated.

7 (a) Is there a clinical diagnostic laboratory attached to the different hospital divisions?

(b) Are there arrangements for post-mortem examination and pathological and bacteriological examinations?

(c) Is there a separate laboratory for haematological and biochemical work on the hospital patients?

(d) Indicate the special types of laboratory tests, e.g., liver function tests, kidney function tests, gastric test-meal analysis, B.M.R., etc., that are commonly undertaken in your hospital.

(e) Are there arrangements for pharmacological testing and determination of toxicity in laboratory animals of unknown medicinal agents?

(f) Is there any arrangement for graphic recording?

(g) Is there any arrangement for radiological examination and physio-therapeutic applications?

8 Is your Institution recognised as a Teaching Centre? If so, what is the number of members of the teaching staff in each department?

9 Is it recognised for post-graduate training? If so what courses of study are offered?

10 Is your Institution prepared to undertake clinical trials on behalf of the Indian Research Fund Association? If in the affirmative please suggest names with qualifications of officers who wish to be on the panel. It may please be stated whether they have undertaken any work of this kind before and details of such work furnished.

11 State the facilities available in your Institute for (a) clinical research and (b) basic research. Would the existing facilities, in your opinion, be adequate for conduct of clinical trials or would further help be needed? If so, a brief statement of the type of help that would be needed may please be given.

12 Is any research work being conducted at your institution with the aid of the funds of the Indian Research Fund Association or any other body or independently?

CHLOROMYCETIN IN RICKETTSIAL INFECTIONS

The efficacy of the antibiotic, chloromycetin, in scrub typhus is likely to prove a discovery of capital importance. The first results of the Anglo-American clinical trial proceeding in Malaya suggests that we at last have a potent remedy for the rickettsial diseases. The antibiotic was isolated in 1947 by Ehrlich and colleagues (*Science*, 106 417, 1947) in Detroit from a soil actinomycete, and its active principle, obtained in crystalline form was found to differ from any antibiotic so far described in containing both nitrogen and non-ionic chlorine. In laboratory infections chloromycetin, weight for weight, appeared more effective against *R. prowazekii* in chick embryos than any other agent tested under these experimental conditions, and large doses produced no symptoms in animals. These encouraging laboratory results demanded clinical trial in human typhus infection, and satisfactory results were claimed in a few cases of epidemic typhus in Mexico early this year. Since March Smadel and other research workers from the U.S. Army and the University of Maryland have been collaborating with Leitch and Soper of Kuala Lumpur in a clinical

trial in scrub typhus, and preliminary results were reported at the International Congress on Tropical Medicine at Washington, D C, in May

Scrub typhus, or tsutsugamushi fever, was one of the major medical problems of the Burma campaign. The disease is indigenous to a large part of tropical and subtropical Asia, but its extent was not realised until large numbers of troops were put into jungle previously unvisited by white men. It has long been recognised in Malaya, and much work has been done on it there by Leithwaite and his colleagues at the Institute of Medical Research, Kuala Lumpur, so it is fitting that this institute was chosen as the headquarters of the trial. The vector of the disease is a mite, not unlike our own "harvester" that picnic parties encounter in the September corn stubble, but picked up in the tropics by walking through the lalang grass. The overgrown rubber plantations are now being cleared after the Japanese occupation and the disease, always endemic, is being encountered again among the native workers. So far 25 patients have been treated with the drug, while a control group of 12 untreated cases have been observed during the same period. The treated and untreated come from the same areas and in some cases from the same plantations, so the strains of *R. tsutsugamushi* are likely to be of similar virulence. The mean ages of the two groups—an important factor in any typhus infection—were the same. The diagnosis was proved in each instance, either by recovering the rickettsia from the blood or by demonstrating satisfactory titres for agglutination against an OXK strain of *Proteus*. In the treated group nobody developed complications or died, the average duration of fever after the first dose was 31 hours, and the average total febrile period 7.5 days, one man, treated on the 3rd day of the disease, was discharged for light work on the 9th day after onset. In the untreated group of 12, 2 patients developed serious complications and 1 of these died, while the mean duration of fever was 18.1 days. The chloromycetin was given by mouth, initially in large doses but these were gradually reduced, and the last 7 cases were given the drug for only 24 hours, receiving a total of 6 gm with an equally satisfactory response. Half the patients were treated on estate hospitals where nursing conditions are necessarily somewhat primitive.

To those who have had experience of scrub typhus in the Burma campaign these results will be more than striking, they will alter the whole picture of a disease. From the laboratory work it is not too much to hope that this new antibiotic will prove equally effective against other rickettsial diseases. If so, the future history of the typhus group of diseases will depend on how far the demand for chloromycetin can be met.—*Lancet*, 1 954, 1948

METHODS OF RAT DESTRUCTION

In the last seven months of 1945, 75 cases of plague were diagnosed in Malta, and Barnet (*J Hyg*

Camb 46 10, 1948) has described the methods used to eradicate the rats carrying the infection. The island has a population density three times that of England, and conditions are favourable for the multiplication of rats, particularly in the towns and urban districts. From June, 1945 to June, 1946, out of 22,902 rats examined 659 were *Rattus rattus* and the remainder *R. norvegicus* (the brown rat). Of this total 20 rats, including 15 *R. norvegicus*, were found to be infected with *Yersinia pestis*. Systematic rat destruction began at the end of August, 1945. The standard rat poisons—zinc phosphide, red squill, arsenious oxide, and the more recent addition, 'Antu' (L-naphthylthiourea)—were used. Plam bait was laid for four or five nights, and poisoned bait on the fifth or sixth night. A fortnight after poisoning a different plain bait was laid to test for residual infestation. Where takes of this bait were observed, baiting was continued again for four or five nights, after which a new poison was added. Cleaning and proofing of buildings were also carried out as far as possible. All farms and built-up areas, including the sewers, were systematically treated. When the control measures started, nearly all the bait was taken, six to seventeen months after treatment only 11.6 per cent of 362 test baits had been touched three days after setting. The reduction of the rat population was accompanied by a decrease in the incidence of plague and of flea-borne typhus (*Research*, 1 334, 1948).

According to O'Connor the rodenticides in common use all have the disadvantage that they are rapidly acting and produce acute symptoms of poisoning even when eaten in sublethal doses. This produces the troublesome condition of "bait shyness" in the rat colony and makes 100 per cent eradication difficult, even when prebaiting is used. He has therefore been studying the use as a rat-poison of dicoumarol, the active principle of spoiled sweet clover, the eating of which has long been known to cause fatal haemorrhagic disease in cattle. The average fatal dose for albino rats weighing 250 g is 28 mg if taken over fourteen days, whereas 30 mg given over three days is not lethal. This necessity for a cumulative effect greatly reduces the danger of accidental poisoning. Dogs weighing 8-10 kg survive single doses of 1 g per kg of body-weight and daily doses of 50 mg administered over ten to fourteen days without developing spontaneous haemorrhages.

For initial field trials dicoumarol was added to bait at the rate of 200 mg per lb dry weight. Prebaiting has proved to be unnecessary, for the poison is entirely acceptable to the wild rat. The most satisfactory technique so far evolved has been to ensure that the rodents have constant and free access to the poisoned bait. The amounts eaten gradually become less as the rats become weaker and die, but the baits should be left for some time to poison the migratory rats which are the source of reinfestation. O'Connor claims that complete clearance can be achieved with dicoumarol more economically than by other methods.—*Lancet*, 1 996, 1948

In Anaemia and Debility

B. C. P. W.

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trial in scrub typhus, and preliminary reported at the International Congress on Tropical Medicine at Washington, D.C., in May

Scrub typhus, or tsutsugamushi fever, was one of the major medical problems of the Burma campaign. The disease is indigenous to a large part of tropical and subtropical Asia, but its extent was not realised until large numbers of troops were put into jungle previously unvisited by white men. It has long been recognised in Malaya, and much work has been done on it there by Lewthwaite and his colleagues at the Institute of Medical Research, Kuala Lumpur, so it is fitting that this institute was chosen as the headquarters of the trial. The vector of the disease is a mite, not unlike our own "harvester" that picnic parties encounter in the September corn stubble, but picked up in the tropics by walking through the lalang grass. The overgrown rubber plantations are now being cleared after the Japanese occupation and the disease, always endemic, is being encountered again among the native workers. So far 25 patients have been treated with the drug, while a control group of 12 untreated cases have been observed during the same period. The treated and untreated come from the same areas and in some cases from the same plantations, so the strains of *R. tsutsugamushi* are likely to be of similar virulence. The mean ages of the two groups—an important factor in any typhus infection—were the same. The diagnosis was proved in each instance, either by recovering the rickettsia from the blood or by demonstrating satisfactory titres for agglutination against an OXX strain of *Proteus*. In the treated group nobody developed complications or died, the average duration of fever after the first dose was 31 hours, and the average total febrile period 7.5 days. One man, treated on the 3rd day of the disease, was discharged for light work on the 9th day after onset. In the untreated group of 12, 2 patients developed serious complications and 1 of these died, while the mean duration of fever was 18.1 days. The chloromycetin was given by mouth, initially in large doses, but these were gradually reduced, and the last 7 cases were given the drug for only 24 hours, receiving a total of 6 gm with an equally satisfactory response. Half the patients were treated on estate hospitals where nursing conditions are necessarily somewhat primitive.

To those who have had experience of scrub typhus in the Burma campaign these results were more than striking, they will alter the whole picture of a disease. From the laboratory work it is much to hope that this new antibiotic will be equally effective against other rickettsial diseases, so the future history of the typhus group of diseases will depend on how far the demand for chloromycetin can be met.—*Lancet*, 1954, 1948

* METHODS OF RAT DESTRUCTION

In the last seven months of 1945, 17 plague were diagnosed in Malta, and Barro

CONTRIBUTION OF RUSSIAN SCIENTISTS
TO ANTIBIOTICS

Pavel Yakimov, Doctor of Chemical Sciences, head of the Penicillin and Antibiotics Laboratory, Leningrad State University writes

The history of penicillin, it is claimed in all Anglo-Saxon textbooks and manuals, begins with that summer morning in 1928 when Alexander Fleming, British scientist, noticed and described the inhibiting influence a spore of green mold *Penicillium notatum* exercises on the development of the yellow staphylococci, bacteria which cause suppurative inflammations

We pay due tribute to the researches of Fleming who observed and described this phenomenon in 1928. We, however, are in duty bound to regard with the same respect analogous investigations conducted by Russian scientists nearly sixty years before Fleming, at the dawn of development of Russian microbiology in Russia

In 1870-1871 two Russian physicians, V. Manassein and A. Polotebnov have established and described in detail the bacterial and curative properties of green mold. A large 72-page monograph with 22 drawings of microscopic preparations entitled "Relation of Bacteria to the *Penicillium glaucom*, Green Mold and the Influence of Certain Means for the Development of the Latter" written by Dr. Manassein was carried in several issues of the Military Medical Journal in 1871

In his extensive experimental study, Manassein for the first time has established—this is repeatedly proved by drawings of microscope preparations—the full disappearance and absence of microbes under the green mold surface. Manassein also drew attention to the fact that the green mold is very unpretentious as regards nutritive media, can oust other types of mold and develop even in solutions of morphine and quinine which are lethal for most other bacteria, which facts are also confirmed by contemporary investigations of Fleming and other scientists

Several issues of another Russian medical journal "Meditsinskiy Vestnik" in 1872 carried a large monograph by Dr. A. Polotebnov entitled "Pathological Significance of Green Mold". In the concluding part of his study D. Polotebnov cites data for a number of experiments he made treating with green mold various types of suppurative ulcers, furuncles, and even syphilis ulcers. The treatment was so effective that he demonstrated his patients to senior students at lectures as well as during visits to the wards. The discovery of the antibacterial and curative properties of green mold by Russian scientists in the '70s of last century is surpassed by far the development of science and medicine of that time. At that period the foundations of scientific microbiology were still being laid and developed by Louis Pasteur. The famous dispute between Pasteur who advanced the microbial theory of fermentation and the scientist Libich who maintained

that fermentation was a purely chemical process caused by the disintegration of proteins was still not settled

In that period when science still had no clear conceptions of the significance and biological role of bacteria, the investigations of the properties of green mold conducted by Russian scientists were so much in advance of the general development of science in these fields that they could not be further developed. Even though Dr. Polotebnov has clearly demonstrated the importance of green mold as a curative means the primitive technique of pharmacology in those days made it impossible properly to develop the findings of the Russian scientists. It was precisely for these reasons that the initial works of Russian scientists, who pioneered in new branches of learning, found no further elaboration

It should be noted that Russian scientists resumed time and again the study of green mold. For example, in 1877 Dr. Lebedinskyy in his dissertation on the etiology of the green mold for the organism noted that though gastric juices reduced substantially the antibacterial properties of green mold, at the same time the number and types of bacteria in the intestinal tract sharply diminished. In 1904 M. Tartakovskiy, a veterinary doctor, published in the "Archive of Veterinary Science" a lengthy article on exudative typhus and chicken cholera in which he describes the swift perishing of the bacteria, of exudative typhus under the influence of green mold which develops on the surface of infected blood. These facts prove the undisputed priority of Russian scientists in the discovery of the antibacterial and curative properties of green mold, subsequently discovered again in Britain in 1928-1940. These separate facts represent only isolated instances in the history of the modern science on antibiotics, which has developed on the foundations of the doctrine (concerning antagonism and struggle among different species of unicelled organisms) evolved by Ilya Mechnikov, great Russian scientist

This is how Louis Pasteur described in 1887 significance of the works of Ilya Mechnikov, then still a young scientist

While all my young collaborators are skeptical about your theory, I at once sided with you, because I myself have for a long time been amazed by the spectacle of struggle between various microscopic organisms which I have had occasion to observe. I think you have landed on the right track.

Mechnikov's subsequent discoveries relating to the struggle among micro organisms have not only brought him immortal fame and added to the glory of Russian science but have also mapped out for many decades the course for further researches

We now are witnessing the triumph of Mechnikov's ideas. The science on antibiotics and antibiotics which is swiftly developing on the foundations of Mechnikov's theory is yielding splendid results

Nearly seventy years ago Mechnikov laid established the highly significant field of inter-cellular

Attempted Isolation of Haem *a* and Porphyrin *a* from Heart Muscle

By J. E. FALK AND C. RIMINGTON

*Department of Chemical Pathology, University College Hospital Medical School,
University Street, London, W.C. 1*

(Received 1 August 1951)

Haem *a* is the name which has been given (Rawlinson & Hale, 1949) to the haem which is apparently the prosthetic group of at least some of the cytochromes *a*. Studies of the visual spectrum of this haem, and of its iron-free derivative, porphyrin *a*, in comparison with the spectra of other porphyrins and haems of known structure (Lemberg & Falk, 1951) have led to the postulation of two possible structures, consistent with all the data available.

In addition to visual spectra an extensive study has been made of the infrared spectra of most natural and many synthetic porphyrins and haems (Falk & Willis, 1951) in the hope that these might provide an analytical tool through which further details of the structure of haem or porphyrin *a* may be made clear. The infrared spectra of porphyrins and haems provide a useful means of identification, not only of individual porphyrins, but also of certain side chains on porphyrins. When the infrared spectrum of pure haem or porphyrin *a* can be obtained, some further light may be thrown on its structure by this means. Unfortunately, the isolation of pure material has not yet been achieved. The study reported below concerns further work on the isolation of the compound, for the purpose of obtaining pure material for measurement of its infrared spectrum and, if it could be obtained in sufficient quantity, for direct chemical study. Haem *a* of doubtful purity has been obtained in very small quantities from horse heart (Negelein, 1933; Roche & Bénévnt, 1936) and from both ox heart and the cells of *Corynebacterium diphtheriae* (Rawlinson & Hale, 1949). The haemochromogen band of this haem, at about 587 m μ ., is easily observed with the hand spectroscope in pyridine extracts of many tissues (heart muscle, pigeon-breast muscle, insect thoracic muscle) after dilution with water and reduction with Na₂S₂O₄. Attempts to isolate the haem are complicated, however, by four factors: (1) The lability of the haem itself, particularly in the presence of tissue components such as cysteine (Rawlinson & Hale, 1949). (2) The concurrent extraction of much protohaem (from haemoglobin, myoglobin, catalase, etc.). (3) The concurrent extraction of a lipid material which it is extremely difficult to remove completely. (4) The relatively minute amount of the haem present in the tissues.

Negelein's (1933) method depended on the extraction of the total haems from water-washed, minced muscle with acetone acidified with hydrochloric acid. The yield of material with 'a very weak protohaemochromogen band at 557 m μ .' was 12 mg. from 5 kg. of fresh mince. The Soret band was at about 430 m μ .

Negelein considered that the haemochromogen of haem *a* had a single visual band (at 587 m μ .). Roche & Bénévnt (1936), on repeating Negelein's procedure, obtained a compound with haemochromogen bands at 587 and 530 m μ . By a modification of Negelein's process they obtained a compound, completely free from protohaem, with only a single band in the visual region, at 587 m μ .; the Soret band was at 425 m μ . Roche & Bénévnt were unable to crystallize this compound satisfactorily; they presented evidence which led them to believe that the compound with the two-banded haemochromogen (587 and 530 m μ .) was the true haem *a*, and the compound with a single visual haemochromogen band an artifact.

In 1949 Rawlinson & Hale developed a new method for the separation of haem *a* from protohaem. After extraction of the haems from the tissues by acetone-hydrochloric acid, they were transferred to ether, adsorbed on a column of aluminium oxide, some lipid removed by washing the column with ether, the haems eluted with hot glacial acetic acid, and transferred again to ether. On extracting this ether solution several times with an aqueous pyridine-hydrochloric acid buffer the protohaem was completely removed, leaving haem *a* in the ether phase. The haem so obtained was contaminated with lipids, but its haemochromogen had only a single absorption band in the visual region (at 587 m μ .). Rawlinson & Hale found that the haem could react with compounds such as cysteine to yield a substance which gave a haemochromogen with visual bands at 553 and 525 m μ ., and that such reactions could occur during isolation by unsatisfactory procedures. They considered, and it now appears acceptable, that the natural haem *a* is the compound which has a haemochromogen with the single visual band (at 587 m μ .). Though Rawlinson & Hale's process was a great improvement on Negelein's, involving far simpler and fewer manipu-

lations, the yield was very small, and the product, which was contaminated by lipids, was unstable. Rawlinson & Hale (1949) prepared a porphyrin from this haem; the visual spectrum of the porphyrin prepared in this manner was used as a basis for some of the work of Lemberg & Falk (1951).

Negelein had earlier (1932*a*) reported the isolation of a porphyrin from pigeon-breast muscle which he called 'cryptoporphyrin'. The haem prepared by the introduction of iron into this porphyrin gave a haemochromogen with bands (about 582 and 531 m μ .) recalling those of some other early haem *a* preparations, and at first he thought that this was possibly the porphyrin of the prosthetic group of the cytochromes *a*. Shortly afterwards, however, Negelein (1932*b*) reported evidence which led him to believe that this porphyrin was an artifact arising from protoporphyrin through the action of hydrochloric acid during the isolation; indeed, in the original paper he reported that the porphyrin could be obtained from crystallized, but not recrystallized, haemin from blood. No cytochrome *a* has ever been identified in blood, and there is thus good evidence that the porphyrin was an artifact. This was further discussed by Lemberg & Falk (1951).

It was now sought, after extraction of the haems from ox heart and conversion of these to porphyrins, to prepare porphyrin *a* in greater quantity and in a pure state. A process was indeed found by which relatively large amounts of porphyrin, free from protoporphyrin, can be prepared conveniently in ordinary laboratory apparatus. It has been shown, however, that porphyrin *a* prepared by this method, and presumably by any method so far available, is a mixture of closely similar substances. Evidence is presented which shows that these substances arise, during the isolation, from one, or at most relatively few precursors.

The cause of the degradation of the original substance(s) has been found to be the action of acid and no process has been found in which this can be avoided entirely. Until such a process is devised, the problem appears to be insoluble.

MATERIALS AND METHODS

Absorption spectra were measured with a Beckman photoelectric spectrophotometer.

Ether was treated to remove peroxides.

Hydrochloric acid concentrations. Because the familiar HCl number (Willstätter no.) widely used in the purification of porphyrins is stated in terms of % (w/v) HCl, this form is used instead of normality.

Reaction with hydroxylamine. To a solution of the porphyrin in pyridine, excess of a mixture of equivalent amounts of solid hydroxylamine hydrochloride and Na₂CO₃ was added, the mixture refluxed gently for 5 min., cooled and filtered.

Preparation of porphyrin *a*. Method A

(1) *Extraction of haems.* Fresh ox heart (4.6 kg.), dissected free of macroscopic fat, yielded 3.2 kg. of minced muscle; this was washed twice with acetone at 0°, pressing out each time, and air dried (800 g.). Of this dried mince, 500 g. were extracted at 3° for 2 hr. with 2 l. acetone containing 40 ml. conc. HCl. The extract was filtered from the tissue residue; so little haem remained in the tissue that a second extraction was not profitable. The filtrate was mixed with an equal volume of ether, and the acetone and HCl washed out with 2% NaCl to minimize emulsions.

(2) *Preliminary defatting.* The ether solution was now run through a column (10 × 3 cm.) of MgO grade III (Nicholas, 1951) packed in ether; the haems were adsorbed as a very deeply coloured layer at the top of the column. The column was then washed with ether (about 2 l.) until the ether running through no longer left a fatty residue on evaporation. The dark zone containing the haems was separated from the column, and the haems eluted with glacial acetic acid. Since MgO dissolves in glacial acetic acid, the elution was quantitative and could be done at the melting point of acetic acid. The acetic acid solution was mixed with about 2 l. ether, and the acetic acid and the magnesium acetate washed out with 2% NaCl; the ether was then removed *in vacuo*.

(3) *Removal of iron from haems.* The residue was dissolved in 100 ml. hot glacial acetic acid, and this solution treated in 20 ml. portions as follows. The haem solution was brought quickly to the boil, and while refluxing gently, about 5 ml. of a boiling saturated solution of ferrous acetate in acetic acid (prepared under CO₂) and 2 ml. conc. HCl were added. The resulting porphyrin solution was cooled as quickly as possible under the tap. This is the process of Warburg & Negelein (1932), modified so as to use the least possible amount of heat.

The several lots of porphyrin solution so prepared were combined, mixed with 2 l. ether, the acetic acid neutralized with sodium acetate, and the ether solution of the porphyrins washed several times with 2% NaCl.

(4) *Removal of protoporphyrin from the porphyrin mixture.* The ether solution was shaken with 500 ml. portions of 4% (w/v) HCl until no more protoporphyrin was removed. This was usually achieved in six or seven extractions; very little porphyrin *a* was extracted at the same time, but most of it remained in the ether phase, where its absorption bands could be seen with the hand spectroscope at 648, 582, 560, 518 m μ . approx. Emulsions were broken when necessary by centrifuging.

(5) *Removal of lipids by treatment with 25% hydrochloric acid.* The ether solution, besides the porphyrin *a*, still contained much lipid material. It was found that this could be quantitatively removed as follows. The ether was removed *in vacuo*, and the residue shaken with 25% HCl at -10°. After standing for about an hour at this temperature the porphyrin was in solution and the lipids which had solidified were easily separated by gravity filtration at -10° (Whitman no. 54 paper). The filtrate was clear and olive-green in colour. Ether was added, the mixture diluted with water, and on neutralization with sodium acetate the porphyrin was transferred to ether. The ether solution could now be washed with water; indeed, after this treatment, no more emulsions occurred at all. No more fatty material could be removed by repeating the 25% HCl treatment. The porphyrin now appeared to be stable if kept in ether or pyridine

solution. No change could be detected by spectrophotometric measurements in material stored for several months at 3°.

From 500 g. dried mince (equivalent to 2000 g. fresh muscle) yields of 18–20 mg. of this porphyrin were regularly obtained in the course of a working day. Its spectroscopic properties were very close to those of the porphyrin prepared by Rawlinson & Hale (1949) (cf. Table 2).

Preparation of porphyrin a. Method B

The haems were extracted from the acetone-dried tissue as in method A, step 1, and gross fat removed as in step 2, except that a column of Al_2O_3 (Savory & Moore) was used instead of MgO , and the haems eluted by several lots of hot glacial acetic acid.

After the elution the haems were again taken into ether, and the ether solution shaken repeatedly with an equal volume of pyridine-HCl buffer (30 vol. pyridine, 0.15 N-HCl to 100 vol.; cf. Rawlinson & Hale, 1949) until no more protohaem remained in the ether phase. The ether solution of crude haem *a* was then evaporated to dryness *in vacuo*. A portion now dissolved in pyridine, diluted with 2 vol. of water, and reduced with $\text{Na}_2\text{S}_2\text{O}_4$ gave a haemochromogen curve identical with the curve published by Rawlinson & Hale (with a single visual band at 587 m μ).

The haem was now dissolved in glacial acetic acid, and the iron removed as in step 3 above; the porphyrin obtained was treated with 25 % HCl as in step 5.

RESULTS

Porphyrin prepared by Method A. Spectrophotometric curves of the material before and after the treatment with 25 % HCl, and of the fatty residue, are shown in Fig. 1. The ratios of the intensities of the absorption bands I–III to that of band IV provide a useful means of comparing such curves (Table 1). As may be seen from Fig. 1, with the removal of the strong absorption in the blue region due to the fat, the intensities of bands I–III increase relative to IV, though the positions of the maxima are hardly changed.

Porphyrin prepared by Method B. This process is essentially the same as that used by Rawlinson & Hale (1949) for the preparation of their porphyrin *a*; the main difference is that instead of the treatment with 25 % HCl they removed some fatty material from the porphyrin by repeated transfers between HCl and ether.

Absorption curves of the porphyrin before and after the 25 % HCl treatment were similar to those shown in Fig. 1. Indeed, the material obtained by

this method behaved in all respects like that from Method A. The manipulations were much more troublesome, however, and the yields much smaller and for most of the experiments reported below material prepared by Method A was used.

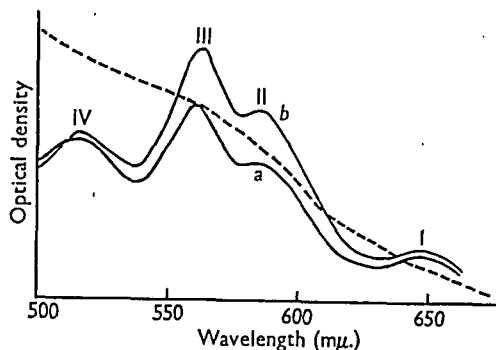


Fig. 1. Visual absorption spectrum of the porphyrin: *a*, before, and *b*, after the treatment with 25 % HCl (stage 5, Method A); ---, the fatty residue. Solvent, pyridine.

Preliminary ether-HCl fractionation of porphyrin a

Rawlinson & Hale observed (personal communication) that the HCl number of their porphyrin apparently became lower as transfers between HCl and ether were repeated. We made similar observations. Thus before the 25 % HCl treatment (step 5) it was possible to remove the protoporphyrin with 4 % HCl (step 4) without appreciable loss of porphyrin *a*. After the treatment, however, even 1 % HCl extracted significant amounts of porphyrin *a*-like material from ether.

We found, on preliminary fractionation of our material, that 6 % HCl removed a considerable fraction, and when no more porphyrin was removed by acid of this strength, a further fraction at least as large could be extracted by 15 % HCl. Absorption data (in pyridine) for typical 6 and 15 % fractions are shown in Table 2, where the measurements of Rawlinson & Hale (1949), calculated to the same form, are included for comparison. The positions of the bands in all the materials were very similar, but band I (about 650 m μ) in the 15 % fraction was more intense than in the fractions extracted by weaker HCl solutions.

It was evident that our porphyrin *a*, which spectrophotometrically was virtually identical with that described by Rawlinson & Hale, was a mixture. It

Table 1. Positions of absorption maxima, and ratios of intensities of the porphyrin (in pyridine solution) *a*, before, and *b*, after the treatment with 25 % HCl (cf. Fig. 1)

Band	...	Positions of maxima (m μ .)				Intensities, relative to band IV			
		IV	III	II	I	IV	III	II	I
	<i>a</i>	517	560	582	647	1.0	1.197	0.852	0.295
	<i>b</i>	517	560	583	648	1.0	1.525	1.148	0.328

Table 2. *Positions of maxima and ratios of intensities of absorption bands of porphyrin preparations (see text); solvent, pyridine*

Band	Positions of maxima (m μ .)				Intensities, relative to band IV			
						IV	III	II	I	IV	III	II	I
Material													
Porphyrin of Rawlinson & Hale (1949)						516	559	582	647	1.0	1.512	1.097	0.301
Porphyrin prepared by Method A						517	560	583	648	1.0	1.525	1.148	0.328
Fraction extracted by:													
6 % HCl						518	562	583	648	1.0	1.530	1.162	0.346
15 % HCl						517	562	584	650	1.0	1.510	1.085	0.523

was at first thought that the material with higher HCl number and increased intensity of band I might be an artifact which had arisen during the manipulations. Artifacts with such characteristics are not uncommon in porphyrin chemistry. Controlled experiments showed that the proportion of this material obtained was not influenced by:

(1) The length of time for which the acetone-dried mince was stored (at 3°) before extraction. There did not appear to be any significant spectrophotometric difference between the product obtained from one half of a batch of acetone-dried ox-heart mince which was extracted at once, and the products from the other half, which was extracted after it had been stored for 24 days at 3°.

(2) The length of time the mince stood with acetone-HCl for extraction of the haems. A batch of acetone-dried ox-heart mince was halved. One half was extracted with acetone-HCl for 1.5 hr. and the porphyrin *a* prepared by Method A immediately. The other half was extracted for 18 hr. and the porphyrin prepared in the same way. There was no significant difference in the spectrophotometric properties, nor in the relative amounts of the porphyrins extractable by 6 and by 15 % HCl in each experiment.

(3) The use of the magnesium oxide or aluminium oxide columns. A batch of the porphyrin was prepared essentially by Method A, the preliminary defatting on the column (step 2) simply being omitted. The procedure was rendered rather more difficult by emulsion formation, but the 25 % HCl treatment removed the fat completely. The product was extracted exhaustively with 6 %, and then with 15 % HCl; two crude fractions were again obtained, their spectrophotometric properties being similar to those reported above (Table 2).

There remained as a possible cause of degradation the treatment with aqueous HCl—at the stages of ether-HCl fractionation, and the 25 % HCl treatment for the removal of fat. Most known porphyrins are quite stable to such treatment, and in addition the strong band at about 650 m μ . was observed with the hand spectroscope in preparations which had never been treated with aqueous HCl (protohaemin having been removed by Rawlinson &

Hale's pyridine buffer treatment). Further, the absorption curves (cf. Fig. 1) before and after the 25 % HCl treatment suggested only that this treatment caused a fall in the absorption at the region of 500–520 m μ . relative to that at about 650 m μ ., and not a specific increase in intensity of the band at 650 m μ .

The hypothesis that the substance of higher HCl number was an artifact was thus not directly proved or disproved.

A possible alternative hypothesis was that it was the substance with lower HCl number and band I of lower intensity which was the artifact. If this were the case, the experiments described above should have provided evidence about it just as well as the original hypothesis they were designed to test. The one process not yet tested was the action of aqueous HCl. The reason which made it appear unlikely that some effect of HCl could have been the cause of the appearance of the substance of higher HCl number (observation of the band before any aqueous HCl had been used) argues not against, but for the possibility that the converse process was taking place, namely, some change was caused by aqueous HCl as a result of which the substance of lower HCl number was derived from the substance of higher HCl number.

Evidence about this was sought by careful fractionation, with HCl, of an ether solution of porphyrin prepared by Method A, and refractionation in the same way of the fractions so obtained.

Full fractionation and refractionation of the porphyrin preparation

An ether solution of the porphyrin (free from protoporphyrin) prepared by Method A was extracted with successive portions of HCl as shown in Fig. 2*a*. The volume of the ether phase was kept constant by the addition of fresh ether as required, and the volume of HCl used at each extraction was equal to that of the ether. The position and intensity of maximum absorption in the Soret region (about 410 m μ .) was determined in each HCl extract. Beer's law was obeyed at the concentrations used, and the density readings were proportional to the porphyrin content of each fraction. For this purpose

it was not necessary to adjust the HCl concentration to the same value in every fraction.

The distribution of porphyrin in the successive fractions is shown in Fig. 2*a*. It is obvious that perfect separation into true fractions was not achieved, but there is good evidence for the presence of several (at least four) components. For the refractionation, the combined 2, 3, 4 and 6 % extracts (*b*), the 8 and 10 % extracts (*c*), and 12, 15 and 20 % extracts (*d*) were transferred to ether, and the ether

(*c*) on refractionation yielded significant fractions to 1, to 8 and to 10 % HCl, but virtually no porphyrin remained after this (fluorescence under ultraviolet light hardly visible). This meant that the fractions which passed from the original into 15 and 20 % HCl were not present in fraction (*c*), nor did they arise during the refractionation. The 1 % fraction of the original should equally have been excluded from fraction (*c*), but apparently more of this material arose during the refractionation. This direction of

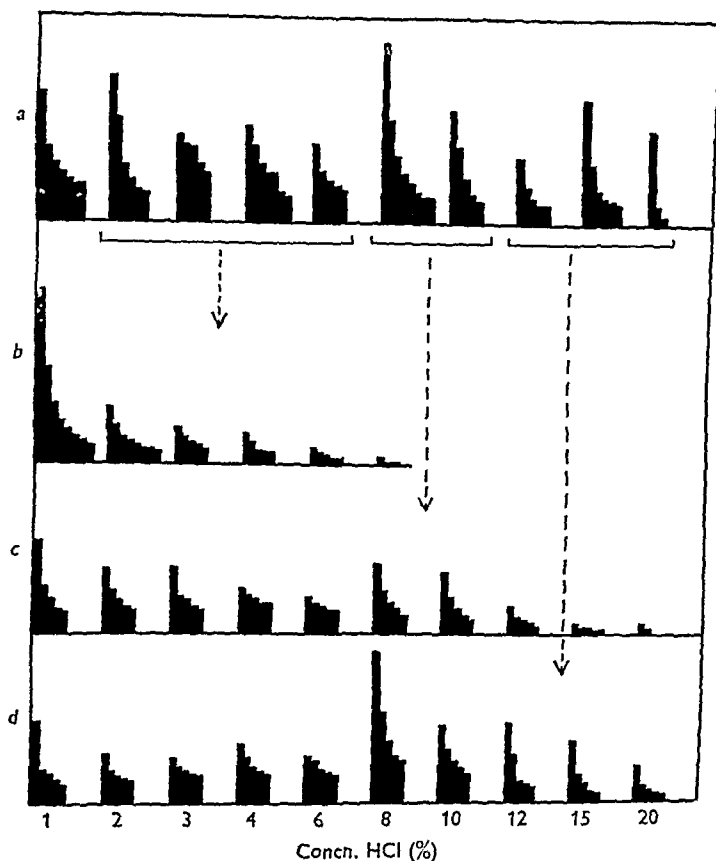


Fig. 2. *a*, the relative yield of porphyrin (determined at the Soret maximum, see text) at successive extractions with HCl. Each step on the histogram represents one extraction with HCl of the concentration shown on the abscissa. *b-d*, the relative yields on refractionation, in the same way, of *b*, the combined 2, 3, 4 and 6 %; *c*, the combined 8 and 10 % (readings $\times 4$); and *d*, the combined 12, 15 and 20 % (readings $\times 8$) fractions from the fractionation shown in 2*a*.

solutions (*b-d*) extracted with successive lots of HCl in the same way as in the original fractionation. The results of these refractionations (Fig. 2*b-d*) were very interesting. It was evident that shaking the ether solutions with aqueous HCl caused a degradation. The direction of this degradation was from products with higher, to products with lower, HCl numbers.

Thus fraction (*d*) could hardly have contained any of the original material which was extracted by HCl concentrations lower than 8 %, yet on refractionation it yielded a pattern of fractions very like that from the original porphyrin solution. Again fraction

the degradation, towards products with lower HCl numbers, is strikingly confirmed by the results of refractionation of fraction (*b*).

Absorption spectra of fractions. The first HCl extract at each HCl concentration (cf. Fig. 2*a*), as the fraction least likely to be contaminated with material of higher HCl number, was transferred to ether, washed well, the ether removed *in vacuo*, and the porphyrin dried and dissolved in pyridine. Absorption curves in the visual region were taken on each of these pyridine solutions; the positions of the maxima and the ratios of the intensities of the bands are shown in Table 3. It was found useful to

Table 3. *Maxima and ratios (in pyridine solution) of the first fraction extracted by HCl at each of the concentrations indicated, in the fractionation shown in Fig. 2a*

First fraction extracted by HCl (%)	Positions of maxima (m μ .)					Intensities, relative to band IV			
	Soret	IV	III	II	I	IV	III	II	I
1	413	512	555	581	645	1.0	1.230	0.900	0.615
2	417	517	560	583	643	1.0	1.660	1.220	0.278
3	417	517	560	582	642	1.0	1.530	1.160	0.316
4	417	516	559	583	645	1.0	1.380	1.078	0.313
6	417	516	561	582	644	1.0	1.338	1.032	0.306
8	418	517	561	583	644	1.0	1.500	1.100	0.310
10	417	518	560	583	645	1.0	1.555	1.150	0.312
12	418	517	561	583	646	1.0	1.560	1.150	0.347
15	418	517	560	584	646	1.0	1.445	1.070	0.345
20	418	517	561	583	649	1.0	1.390	1.050	0.420

introduce a graphical method for comparison of the spectroscopic properties of these rather similar materials. The changes in position of the maxima are readily appreciated from a consideration of the table, but the pattern in the ratios of intensities of the bands in the different fractions is made much clearer by plotting them as in Fig. 3.

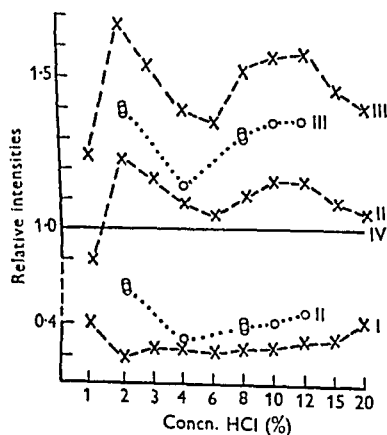


Fig. 3. Diagram showing the change of intensities of bands I-III relative to band IV; data from Table 3. The relative intensities after reaction with hydroxylamine are shown as \circ .

The similar data from the refractionation of fractions (b) and (d) are shown in Fig. 4, in which the pattern of the ratios of intensities of the bands in the original fractions is strikingly reproduced. This similarity leaves no doubt that really different materials are contained in these different fractions.

It is noteworthy that all the compounds from refractionation of fraction (d) (Fig. 4a) have the relative intensities of bands II and III depressed in comparison with the corresponding original fractions (Fig. 3). For example, the porphyrin extracted by 6% HCl (Fig. 3) has a true oxorhodo type spectrum, while the 6% fraction (Fig. 4a) has only a weak rhodo type spectrum. The accuracy of the

determination of the ratios relative to band IV was approximately ± 0.02 unit for bands II and III and ± 0.05 unit for the much weaker band I. Band I was significantly more intense in the 15 and 20 % fractions, and this increased intensity was paralleled by a slight shift to longer wavelengths. This was the only consistent marked change in the positions of the maxima.

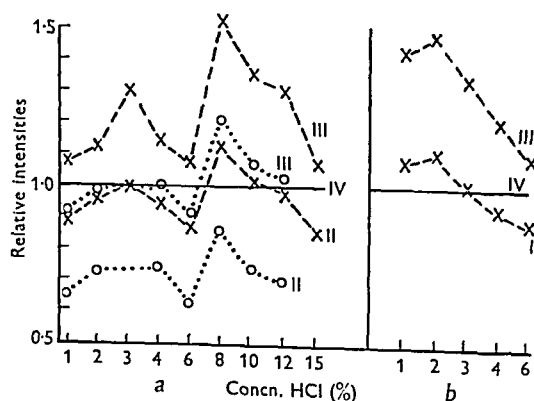


Fig. 4. The changes in relative intensities in the materials from: a, stage d, Fig. 2; b, stage b, Fig. 2. Relative intensities after reaction with hydroxylamine are shown as \circ .

Fractionations in the above manner were carried out on several samples of porphyrin, prepared by Method A from different batches of ox hearts, with consistent results.

Effect of heating the porphyrin with aqueous hydrochloric acid

Since it appeared that the changes were caused in some way by aqueous HCl, its effect was studied in more detail. It was hoped that the material might be degraded by HCl under more drastic conditions to a useful, single degradation product.

In a preliminary experiment some material prepared by Method A was fractionated as in Fig. 2a above. The combined 8 and 10 % fractions, and the combined 12, 15 and

20% fractions were transferred to ether, the solution washed, the ether removed, and the residues dissolved in 25% HCl. These HCl solutions were each divided into four portions. Portion 1 was at once transferred to ether, washed,

pected to shift towards shorter wavelengths; there were not any significant changes in the positions of the other bands. The changes in the relative intensities of the bands were, however, quite marked and these are shown in Fig. 5.

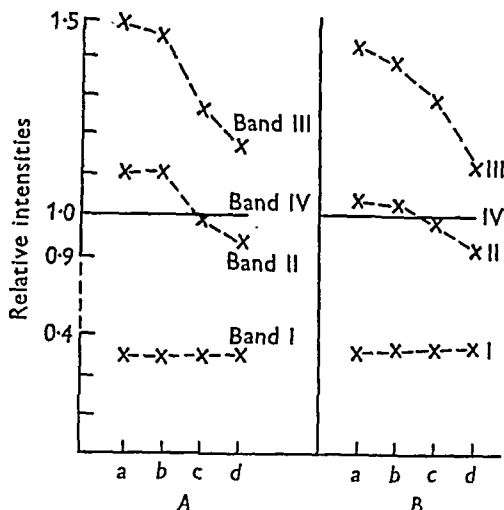


Fig. 5. The change in intensities of bands I-III relative to band IV, when: *A*, the combined 8 and 10% fractions and *B*, the combined 12, 15 and 20% fractions (Fig. 2*a*) stood in the dark in 25% HCl solution *a*, at zero time; *b*, after 50 hr. at 10°; *c*, after 30 hr. at 30°; and *d*, after 3 hr. at 70°.

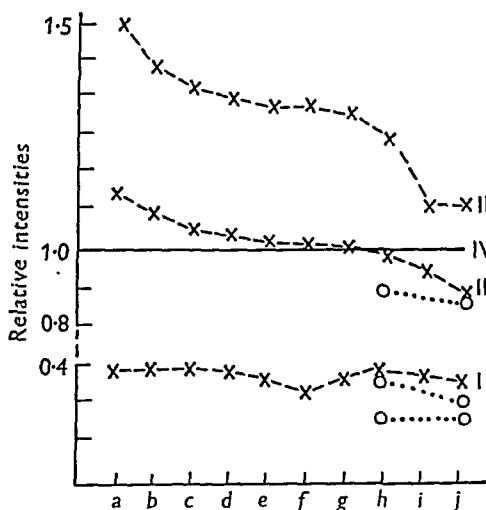


Fig. 6. The change in relative intensities of the bands of porphyrin prepared by Method *A*, after standing in the dark in 25% HCl solution *a*, at zero time; *b*, after 50 hr. at -10°; *c-h*, after 1, 2, 3, 4, 5, 6 hr. at 57°; *i, j*, after 1.25 and 5 hr. at 95°. The relative intensities after reaction with hydroxylamine are shown as -O-.

the ether removed and an absorption curve measured on the residue dissolved in pyridine. Portion 2 was allowed to stand in the 25% HCl at -10° for 50 hr.; portion 3 stood in the dark at 30° for 30 hr. and portion 4 stood in the dark at 70° for 3 hr. At the end of these times each sample was transferred to pyridine and its absorption curve measured. In the small samples used, band I was too weak for accurate determination of its position, which might have been ex-

pected to shift towards shorter wavelengths; there were not any significant changes in the positions of the other bands. The changes in the relative intensities of the bands were, however, quite marked and these are shown in Fig. 5.

It is seen that the 25% HCl, even at -10°, caused a depression of the intensities of bands II and III relative to that of band IV. The depression of these bands was increased and hastened as the temperature increased. These measurements were on the whole samples, without fractionation. The depression of the bands in the separate fractions (Figs. 3 and 4*a*) was paralleled in the present experiment by the depression of the bands in the unfractionated material.

In another experiment, porphyrin prepared by Method *A* was treated, in HCl solution, as indicated in Fig. 6. The material, which in this experiment had been treated at 57° for 6 hr., was transferred to ether and fractionated with HCl. The pattern of relative intensities of the absorption bands of the fractions was similar to that shown in Fig. 4*a*. As in the previous experiment, the depression of the ratios of bands II and III increased both with time and temperature, though the band positions hardly changed. Even treatment of a 25% HCl solution at 95° for 5 hr., however, led to a change only from oxorhodo- to rhodo-type spectrum, band I remaining at 644 mμ.

Reactions with hydroxylamine

Various fractions were treated with hydroxylamine, and the spectroscopic properties of the products are indicated in Figs. 3, 4*a* and 6. It may be seen that in all fractions so treated, irrespective of the character of the spectrum before the treatment, oxime formation had taken place, demonstrating that the -CHO group was still intact. This is discussed below.

Evidence for degradation during removal of iron (step 3, Method A)

There is no doubt that HCl, during the HCl-ether fractionations, and also during the treatment with 25% HCl causes changes in the material. It appeared likely that similar changes would occur during the removal of iron.

Porphyrin mixtures after removal of iron from the haems were transferred immediately to ether and esterified with diazomethane. Chromatography of the esters on columns of aluminium oxide grade IV and of magnesium oxide grades III and IV (cf. Nicholas, 1951) showed the presence, apart from protoporphyrin, of a variety of porphyrin *a*-like materials which could not be satisfactorily resolved. It is only necessary to report briefly that materials with spectra similar to most of the fractions shown

in Fig. 3 were obtained from the chromatograms. It thus appeared that, during the removal of iron, materials similar to those which arise during treatment with HCl had appeared.

Attempts to purify haem a

There is no evidence yet that the haem, as such, is labile in acid conditions. This was borne out to some extent when, instead of acetone-HCl, cold pyridine was used to extract the haems from the tissue. The haems were transferred to ether, and without any attempt at removal of the large amount of lipid material the protohaem was removed by the aqueous pyridine-HCl buffer method of Rawlinson & Hale (1949). The haem *a* so obtained was grossly contaminated with fat, but its haemochromogen had only a single visual absorption band, at 587 m μ , apparently the same as the material prepared after acetone-HCl extraction of the tissue followed by elution from alumina by boiling acetic acid. This haem was eventually converted to porphyrin, and this treated by Method A. On fractionation of an ether solution of this porphyrin with HCl, fractions identical with those shown in Fig. 2*a* were obtained.

Chromatography of haems

The haems were extracted from the tissue and defatted as in steps 1 and 2 of Method A. The resulting ether solution of the haems was evaporated to dryness *in vacuo*. Neither light petroleum nor benzene extracted any fatty material from the dry residue. The dry material was soluble in butanol, which also dissolved the haem when shaken with a suspension of it in water brought to pH 4. The dried haems, or these, after esterification with diazomethane, were used for the following experiments.

The haem esters were chromatographed on columns of the following absorbents: Alumina grades II and IV, magnesium oxide grades II and III (cf. Nicholas, 1951); talc and kieselguhr (Hyflo supercel). The following solvents were used, singly and in pairs, in varying proportions: chloroform, methanol, ether, benzene, pyridine. Separation of both the haems and the haem esters was also sought on partition columns. The following solids were tried as supports: filter paper powder (Whatman, standard grade) and kieselguhr (Hyflo supercel). For 'reverse phase' chromatography, Hyflo treated with dichlorodimethylsilane was used (cf. Martin, 1949; Howard & Martin, 1950). The solvent systems tried for all three supports were lutidine-water, equilibrated and used at both 21° and 3° and other-pyridine buffer (pyridine 30 vol., 0.15N-HCl to 100 vol.), at room temperature (18°).

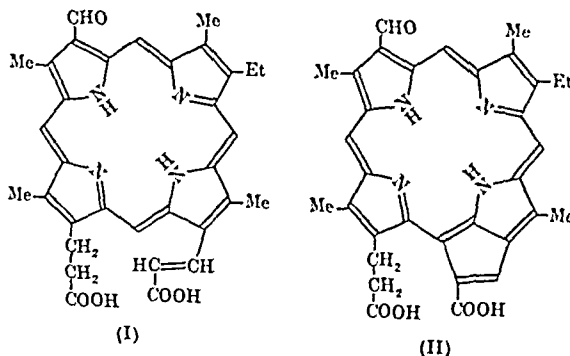
There was no indication in any of these experi-

ments that the haem *a* might be separated from the other materials. The coloured material in every case moved slowly down the column with the solvent front. Material which eventually ran through the columns had the same proportion of haem *a* to protohaem as the starting material.

DISCUSSION

From the experiments reported, it became clear that once the iron is removed from haem *a*, the porphyrin is very unstable in the presence of acid. The spectrum of the product obtained was the sum of the spectra of the degradation products, and it is clear that the proportions of these, and the resulting mixed spectrum, vary with both time and temperature during manipulations with acid.

The tendency of the material with higher hydrochloric acid number and band I at longer wavelengths to be changed to material with lower hydrochloric acid number and band I at shorter wavelengths, as well as the constant downward trend in the intensities of bands II and III relative to band IV, are consistent with the hypothesis that the



changes are due to the destruction of 'rhodofying' groups (Lemberg & Falk, 1951). Among such groups are the $-\text{CHO}$ group, the $-\text{CH}:\text{CH}:\text{COOH}$ group (Formula I), and the unsaturated isocyclic ring (Formula II). These were suggested by Lemberg & Falk (1951) as possible structures for porphyrin *a*, consistent with the visual spectroscopic properties of haem *a* and porphyrin *a* in comparison with the properties of compounds of known structure. As a basis for this study, the spectra of haem *a* and porphyrin *a* described by Rawlinson & Hale (1949) and Rimington, Hale, Rawlinson, Lemberg & Falk, (1949) were used. The presence of a $-\text{CHO}$ group was confirmed (Lemberg & Falk, 1951), but the exact nature of the other 'rhodofying' group is not known. These studies are considered not to be invalidated by the present work, since it seems likely that the undegraded, natural porphyrin *a* has spectroscopic properties very close to those of Rawlinson & Hale's (1949) material.

Since it is not clear how a $-\text{CHO}$ side chain could be changed by HCl in such a way as to yield products with the characters described above, it was considered more likely that it was the other 'rhodofying' group which was being changed. That this was so became apparent when it was found that the material which had been heated to 95° for 5 hr. in 25% hydrochloric acid solution was still able to react with hydroxylamine, the spectrum changing from rhodo to aetio type and band I shifting from 647 to 636 $\text{m}\mu$.

These findings were parallel with those of Rawlinson & Hale (1949) and Rimington *et al.* (1949), who found that treatment of their porphyrin *a* with diazoacetic ester or HI (double bonds in side chains) or hydroxylamine (carbonyl groups in side chains) led to a change from oxorhodo- to rhodo-type spectrum, band I hardly shifting in position. The action of both these types of reagent in succession, however, led to a product with aetio-type spectrum and band I at 625 $\text{m}\mu$. The parallelism between the spectra of numerous fractions before and after reaction with hydroxylamine (Figs. 3, 4a, 6) showed, moreover, that the effect of the $-\text{CHO}$ group on the spectrum was approximately equivalent in all the materials. Thus the differences between the fractions must be due to a series of changes in the other rhodofying group. It is evident that this group is gradually changed by acid towards an end state in which its rhodofying effect is completely lost. It is not possible, however, to postulate intermediate steps in this degradation which could account for the many apparent stages in the change.

Acid apparently caused degradation of the porphyrin even at step 3 (removal of iron). It is possible that the numerous products revealed by chromatography directly after this step were original components, but their similarity to the materials which were shown to be produced by hydrochloric acid at later stages makes it more likely that they arose in the same way. All methods for removing metals from metalloporphyrins (including the relatively mild method of Paul, 1950), feature strongly acid conditions except the sodium amalgam method (Fischer & Hilger, 1924). The latter was unsuitable, however, because the $-\text{CHO}$ side chain and the side chain with an ethylenic double bond would be reduced. Thus no suitable alternative process is available for this step, nor could any be found for the other steps involving the use of acid. Fractionation of ether solutions of the haems with aqueous NaOH , Na_2CO_3 or Na_2HPO_4 was ineffective. Until suitable techniques are developed for all these steps, attempts at purification through the porphyrins must be unsuccessful.

It appeared more profitable to turn again to the separation and purification of the haem as such. There is no direct evidence that the haem is unstable

in acid conditions, though this possibility cannot be ignored. It should be pointed out that the haemochromogen band at 587 $\text{m}\mu$. in haem *a* prepared by Rawlinson & Hale's (1949) method, and, indeed, in direct pyridine extracts of tissues, is broad, and may include the bands of several similar compounds. It is quite possible that there exists more than one natural haem *a*, perhaps corresponding to different cytochromes *a*. Examination at very low temperatures (*cf.* Keilin & Hartree, 1949) of this haemochromogen band in pyridine extracts of tissues might allow the detection of such components, though the lipids extracted concurrently by pyridine would make such a study difficult.

There is little doubt that the failure to separate the haems by chromatography was due to the presence of lipids, which might be expected to change their partitioning properties. The gross fat can be removed without much trouble, but the lipid which is encountered in smaller but appreciable quantities in attempts to purify haem or porphyrin *a* is still, perhaps, the greatest single factor hindering its isolation.

It appears now that the most fruitful approach to the problem might be to abandon the efforts to purify the natural compounds and instead to attempt to prepare a stable derivative of the haem through, for example, catalytic hydrogenation, fusion in resorcinol or the action of diazoacetic ester. Material obtained by the procedures of steps 1 and 2, Method A, may be sufficiently free of gross contamination for this purpose. Unfortunately, it is not certain that the haem even at this stage has not already suffered some change.

SUMMARY

1. During attempts to find a method for the preparation, from ox-heart muscle, of porphyrin *a* in quantities sufficient for direct chemical study, it was found that the porphyrin is very unstable in acid media.

2. Evidence is presented that the $-\text{CHO}$ side chain of the porphyrin is not changed during this degradation. It appears that the other 'rhodofying' group is modified in a gradual manner, leading to a series of porphyrins with rather similar spectra. The degradation is hastened at raised temperatures, and in the end-state the 'rhodofying' properties of the group are lost completely. It was suggested (Lemberg & Falk, 1951) that this group may be an acrylic acid side chain. It is not yet possible, however, to interpret in terms of chemical structure the changes which occur during the degradation.

3. No procedure has been found by which the use of acid can be avoided entirely during the isolation of the porphyrin.

4. Haem *a*, as such, may not be unstable to acids, though there is no direct evidence on this point.

Attempts were made by the use of both absorption and partition chromatography to find a method for the isolation of relatively large amounts of haem α , but without success.

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Sydney and Melbourne respectively are collaborating in this programme, and with Prof. D. Keilin, F.R.S.

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Some Properties of the Glutaminase of *Clostridium welchii*

BY D. E. HUGHES AND D. H. WILLIAMSON

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, the University, Sheffield 10

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In previous papers from this laboratory (Krebs, 1948; Hughes, 1949, 1950) it was reported that cetyltrimethylammonium bromide (cetavlon) accelerates the decarboxylation of glutamine and glutamic acid in intact cells and extracts of *Clostridium welchii*. The present paper is a study of the mechanism of this effect. The glutaminase has been purified and the effect of cetavlon upon purified enzyme preparations has been investigated. Whilst cetavlon accelerates the rate of deamination of glutamine in intact cells and crude extracts it inhibits it in the purified extracts. These findings and the result of kinetic studies support the view put forward previously (Hughes, 1949) that the accelerating effect of cetavlon is due to the removal of an intracellular inhibitor normally accompanying the enzyme.

METHODS

Organisms. Three strains of *Clostridium welchii* (strains SR 12 and 1490 of the National Collection of Type Cultures, and a locally isolated strain) were maintained in Robertson's meat medium. Through the courtesy of Dr B. C. J. G. Knight, two batches of about 500 g. (wet wt.) of strain 1490 were made available from the Wellcome Physiological Laboratories, Beckenham, Kent. These cells had been collected after 5 hr. growth on the papain digest described below.

Growth medium. The usual medium consisted of casein hydrolysate, meat, yeast extract and glucose (see Krebs,

1948). The papain digest meat medium was prepared according to Ainsworth, Brown, Marsden, Smith & Spilbury (1947). A semi-synthetic medium was prepared from hydrolysed casein (McIlwain & Hughes, 1944) as described by Boyd, Logan & Tytell (1947).

Measurement of enzyme activity. In general, the activity of the glutaminase was estimated by determination of the rate of ammonia formation. A fresh solution (0.5 ml.) of glutamine (0.02 M in 0.25 M-sodium acetate buffer containing 0.025 M-KCl) was placed in one arm of a branched test tube made from 20 mm. diameter Pyrex tubing in the form of an inverted Y. The other arm contained 1.5 ml. of the enzyme solution in acetate buffer (final concn. 0.2 M) and KCl (final concn. 0.025 M). A series of parallel tubes was placed in a water bath maintained at 40.0°, and after 1 min. equilibration the contents of the two arms were mixed without removing the tubes from the bath. At 5 min. intervals the tubes were removed from the bath and the reaction stopped by placing in ice water and adding 0.5 ml. N-H₂SO₄. NH₃ was determined according to Parnas. Blank NH₃ determinations were made on all reagents. In this way a time curve of the glutaminase activity was obtained. The initial rate of NH₃ formation was linear in intact cells and in crude extracts of *Cl. welchii* until approximately 50–60% of the added 224 μ l. of glutamine was decomposed, except where the glutaminase activity was low, i.e. where less than 5% of the substrate was decomposed in 15 min. The enzyme dilution was therefore adjusted so that not more than 60% and not less than 10% of the glutamine was decomposed in 15 min. Under these conditions the initial rate of reaction was proportional to the dilution of the enzyme. Duplicate

estimations of enzyme activity agreed within $\pm 5\%$. The $q_{NH_3}^N$ (i.e. $\mu\text{l. NH}_3/\text{mg. total N in enzyme preparation/hr.}$) was estimated from the linear part of the curve.

Chemical estimations. Total N was determined by micro-Kjeldahl method (digestion with H_2SO_4 and copper selenate for 1 hr.). Non-protein N was determined on solutions after precipitation with 3% trichloroacetic acid and protein N by difference. Chloride was estimated by the colorimetric method of Conway (1947).

Chemicals. The L-glutamine, L-glutamic and cetavlon were the same specimens used in a previous paper (Hughes, 1950). The safranin, phenolphthalein and sulphonphthalein dyes were commercial specimens.

Extraction of the enzyme

Solutions of glutaminase can be prepared by grinding the cells with powdered glass and extracting the ground mass with buffers (Hughes, 1949). This method was inconvenient for cell quantities exceeding 3.0 g. wet wt. The setting free of the enzyme by autolysis, which was successful in the case of the glutaminase of *Proteus morganii* (McIlwain, 1948), was unsuccessful as most of the enzyme activity was lost. It was found, however, that the glutaminase was extracted almost quantitatively by phosphate or borate buffers, pH 7.5–8.5, from cells dried *in vacuo* from the frozen state. A batch of 85 g. (wet wt.) cells was dried without loss of glutaminase activity in a desiccator of 8 in. diameter and 10 in. high, as follows: a paste of cells obtained by centrifugation at 3500 g. for 15 min. was spread in 1–2 mm. layers in Petri dishes (6 in. diam.) and frozen in the freezing compartment of a refrigerator. The dishes were then stacked between dishes containing P_2O_5 , from which they were separated by glass triangles, leaving spaces for ventilation. The desiccator was maintained continuously at 0.5–0.1 mm. Hg by an oil pump. The heat generated by the reaction of the P_2O_5 with the water vapour from the cells was sufficient to thaw the surface layer of the cell paste. As the P_2O_5 became liquid it was replaced with dry P_2O_5 until it no longer adsorbed water, showing that the cells were almost dried. During the first hour P_2O_5 had to be replaced at 15 min. intervals and three or four times afterwards. After 10–12 hr. the evacuated desiccator was placed at 2° for 2 days, after which the dried cells were finely powdered in a pestle and mortar and stored at 2° in an air-tight container. The dried powder lost no more than 15% of its glutaminase activity after 7.5 months' storage.

To extract the enzyme, the dried cells (10 g.) were mixed with 60 ml. of 0.05 M- Na_2HPO_4 containing 0.025 M-KCl and 15–20 glass beads (5 mm. diam.) in a stoppered conical flask.

The flask was rotated mechanically at about 10–15 rev./min. for 15 hr. at 2° , the resulting sludge was centrifuged and the bulk of the supernatant was collected by decanting. The residue was mixed with 40 ml. Na_2HPO_4 -KCl buffer, centrifuged and washed twice with 30 ml. of the above buffer. Supernatant and washings were combined and centrifuged at 4000 rev./min. for 30 min. The viscous grey-green supernatant solution contained 80–90% of the original glutaminase activity (Table 1).

Purification of the enzyme

The steps used in the purification were: fractional precipitation with acetic acid (stages 1 and 2); treatment with safranin (stage 3); dialysis (stage 4). The details of the purification of an extract from 10 g. of strain 1490 are as follows:

Stage 1 (first acid precipitation). The clear extract (80 ml.) obtained as previously described was cooled on ice, and acetic acid (10%) was added slowly with stirring. When the pH reached 4.0 (checked by glass electrode) addition of acetic acid was stopped and stirring continued for a further 15 min. The precipitate was collected by centrifuging and washed twice in 0.05 M-acetate buffer, pH 4.0. The supernatant and washings were discarded. The precipitate which was at first very viscous, became firm and the centrifuge tube was inverted and drained. The solid was extracted with 105 ml. of 0.2 M- Na_2HPO_4 containing 0.025 M-KCl, the undissolved residue which was inactive was removed by centrifuging and discarded.

Stage 2 (fractionation at pH 5.0 and 4.1). The solution from stage 1 was cooled on ice and stirred while acetic acid (2%) was added slowly until the pH fell to 5.0. The precipitate which contained about 25% of the total enzyme activity and 50–60% of the dry weight of the precipitate from stage 1 was removed by centrifuging and discarded. Acetic acid was added to the supernatant until the pH fell to 4.1, stirring continued for an hour, after which the precipitate was collected by centrifuging and washed twice in 100 ml. of 0.05 M-acetate buffer, pH 4.1, containing 0.02 M-KCl. After washing, the precipitate formed at pH 4.1 was dissolved in 35 ml. of 0.02 M- Na_2HPO_4 containing 0.025 M-KCl and a slight amount of insoluble material removed by centrifuging. Acetic acid (2%) was added to the solution until the pH was 4.0, the precipitate collected by centrifugation and dissolved in 30 ml. of 0.02 M- Na_2HPO_4 containing 0.1 M-KCl; it gave a clear solution (pH 8.2), containing the glutaminase.

Table 1. Partial purification of the glutaminase of *Clostridium welchii* strain 1490

(Experimental details are described fully in the text.)

Preparation	Volume of preparation (ml.)	Total N (mg.)	Activity N ($q_{NH_3}^N$)	Yield (%)
Intact cells (52 g. wet wt.)	—	1100	3500	—
Cells of <i>Cl. welchii</i> NCTC 1490 from the Wellcome Laboratories frozen and dried over P_2O_5 (wt. = 10 g.)	—	1100	3400	97
Combined extract and washings	80	685	4300	88
Stage 1, first acid precipitation	120	336	6800	69
Stage 2, precipitate at pH 5.0	60	210	4450	27.8
Second precipitate at pH 4.1 (F3)	32	68.4	17300	38
Stage 3, filtrate from Zeo-Karb 216	66	34.0	44000	44
Stage 4, above filtrate after dialysis	60	15.6	83000	40.5

With other batches of cells, preliminary tests had to be carried out on the solubility of the enzyme and the procedure modified accordingly. The first precipitate at pH 5.0 in the case of older cells (16 hr.) containing 50–60% of the activity (compared with 20% in younger cells) was redissolved in Na_2HPO_4 and taken to pH 5.0 with acetic acid. The resulting precipitate was discarded and the two mother liquors combined and taken to pH 4.2. The precipitate containing 70–80% of the activity was redissolved and precipitated at pH 4.0 and worked as before. A reprecipitation was necessary in the case of extracts from still older cells (25–48 hr.).

Stage 3 (treatment with safranin). Geddes & Hunter (1927) introduced safranin as a reagent in the purification of enzymes. The method which they used for the asparaginase of yeast was modified by changing the safranin concentration and by working at pH 8.3 instead of at near pH 6.0. This treatment precipitated the bulk of the inert material whilst the glutaminase remained in solution. The solution (32 ml.) resulting from stage 2 was cooled in ice, and stirred while 35 ml. of a solution of safranin (0.5% in 0.2M- NaHPO_4 containing 0.025M-KCl) were slowly added. Stirring was continued for 15 min. and a sample of the solution was centrifuged and tested with safranin. Further quantities of safranin solution were added in three 5 ml. portions until tests showed that precipitation was complete. After 15 min. standing the bulky red precipitate was removed by centrifugation. The red supernatant was cooled to 2° and filtered by gravity twice through the same column of Zeo-Karb 216 (10 mm. diam., 200 mm. long). This treatment removed the safranin; the remaining colour of the solution was a faint yellow.

Stage 4 (dialysis). The filtrate was dialysed against 2000 ml. 0.04M-KCl in a stirred system, for 48 hr. at 2°; the KCl solution was then changed and dialysis was continued for another 16 hr. A precipitate which had formed was removed by centrifuging and pH was adjusted to 7.0 with acetic acid. The clear solution was then placed in test tubes in 8–10 ml. portions, frozen on solid CO_2 and stored at -15°. This preparation was used in the following experiments on the properties of glutaminase. The $q_{\text{NH}_3}^{\text{N}}$ of one batch was 83000 and the yield about 40% (Table 1). Similar material was prepared by this method from dried cells of strain SR 12 and the locally isolated strain.

RESULTS

The kinetics of glutaminase

pH-Activity curve. Acetate buffers (pH 3.5–5.6), lactate buffers (pH 2.5–3.5) and veronal-citrate-phosphate buffer, pH 2.5–6.0 (Britton & Robinson, 1931), were used. The nature of the buffer made no difference to the pH curve, nor did the variation of the concentration of the acetate buffer from 0.02 to 2.0M over the pH range tested (4.1–5.6). The slope of the pH curve, however, changed with the purification. The optimum pH remained unchanged at pH 5.0–5.2, but the optimum was usually broader with purer preparations (Fig. 1). The differences

between the activity of pure and crude preparations were greater at low pH values. The change in pH curves upon purification is consistent with the suggestion that inhibitors of glutaminase are present in intact cells and crude extracts (Hughes, 1949).

Temperature and rate of deamidation. The temperature coefficient of deamidation by purified glutaminase between 15 and 40° was 1.8–2.0. At 60° the enzyme was inactivated too rapidly for the rate of deamidation to be measured.

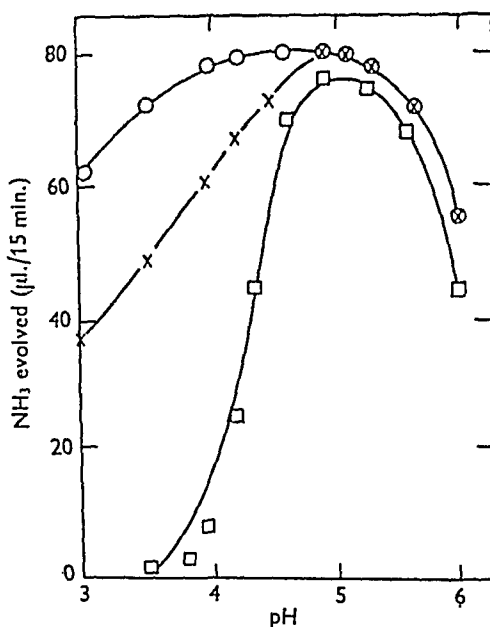


Fig. 1. The pH curve of some glutaminase preparations. Each tube contained: 0.005M-glutamine; Britton & Robinson (1931) buffer; 0.025M-KCl; enzyme preparation sufficient to evolve 70–80 $\mu\text{l. NH}_3/15 \text{ min.}$ at pH 5.0. \bigcirc — \bigcirc , purified enzyme; \times — \times , intact washed cells of strain 1490 and SR 12; \square — \square , extracts of strain SR 12 prepared from cells ground with glass.

Progress of deamidation and degree of purification.

Under standard test conditions the rate of reaction was linear until 85–90% of the substrate was decomposed (Fig. 2) if the purified enzyme was used. With cruder preparations, however, as already stated, the rate of reaction fell off earlier (Fig. 2). The cause of these differences will be discussed later. The quantities of ammonia and glutamic acid were identical at all stages of the reaction (Table 2). There was no evidence of the formation of pyrrolidone carboxylic acid.

Attempts to reverse the reaction by addition of adenosinetriphosphate (ATP), magnesium, phosphate (Speck, 1949; Elliott & Gale, 1948; Fry, 1949) were unsuccessful. Neither intact cells nor extracts formed glutamine from ammonium glutamate under these conditions.

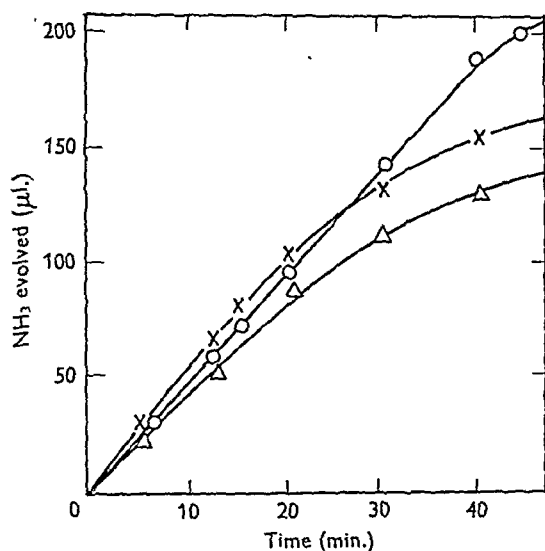


Fig. 2. Progress of the reaction with various glutaminase preparations. Each tube contained in a final volume of 2.0 ml.: 0.005M-glutamine; 0.2M-acetate buffer, pH 5.0; 0.025M-KCl; and enzyme solution. x—x, intact cells; Δ—Δ, acid-precipitated enzyme; O—O, purified glutaminase.

Table 2. *The rate of glutamic acid and NH₃ formation by purified glutaminase*

(Each tube contained in a final volume of 2 ml.: 0.005M-glutamine; 0.2M-acetate buffer, pH 5.0; 0.025M-KCl; 0.5 ml. enzyme solution containing 0.004 mg. total N. All concentrations as final concentration. NH₃ was estimated as described on p. 45 and glutamic acid and glutamine by a modification of the method of Gale (1947), see p. 49.)

Time of reaction (min.)	NH ₃ (μl.)	Glutamate (μl.)	Glutamine (μl.)
0	—	3	218
5	29.0	23	—
15	109	110	113
25	161	160	—
35	210	209	3.0

Effect of substrate concentration. The substrate concentration activity curve of purified glutaminase (Table 3) obeyed the Michaelis-Menten equation. The results gave a straight line when plotted according to Lineweaver & Burk (1934), i.e. as a

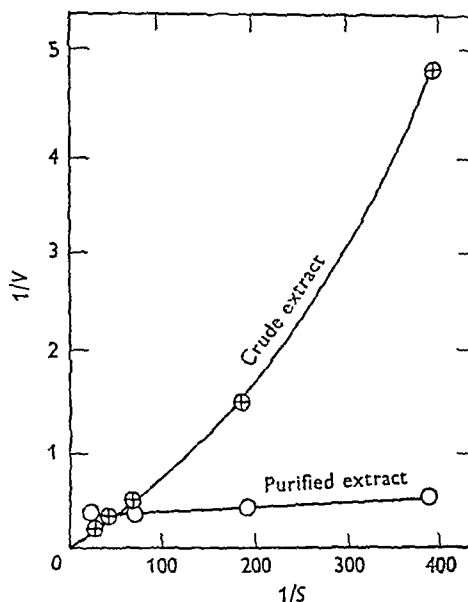


Fig. 3. Effect of purification on the initial velocity of deamidation at varying substrate concentrations. The conditions of each experiment were as described in Table 3, but the pH was 4.1 instead of 5.0. Ordinate $1/v \times 100$ for purified glutaminase and $1/v \times 10$ for the crude extract prepared from cells broken with powdered glass; s = molar concn.; v = μ l. NH₃ evolved/5 min.

reciprocal of velocity against the reciprocal of substrate concentration. The Michaelis constant as calculated from such plots was from 0.0006 to 0.00063M. Intact cells and crude extracts gave curves that did not follow strictly the Michaelis-Menten equation (Fig. 3). The half-maximum rate with these materials was at 0.012–0.001M-substrate concentration. Thus the apparent affinity of the enzyme for its substrate increased upon purification.

Table 3. *Rate of deamidation by the purified enzyme at varying substrate concentrations*

(Each tube contained glutamine; 0.2M-acetate buffer, pH 5.0, and purified glutaminase (0.003 mg. total N/ml.). All concentrations are as final concentrations. NH₃ in the contents of each tube was estimated by the standard method).

Enzyme added (ml.)	Glutamine concentration (M)	Total vol. (ml.)	NH ₃ /5 min.	
			(μl./total vol.)	(μl./2.0 ml.)
1.0	0.01	4.0	64.0	32.0
1.0	0.005	4.0	61.0	30.5
1.0	0.0025	4.0	61.0	30.5
1.5	0.0017	6.0	73.4	26.7
2.0	0.0013	8.0	87.7	21.9
2.5	0.0010	10.0	102.0	20.5
2.5	0.0005	10.0	76.0	15.2

Stability. Purified glutaminase lost no activity when stored at -10 to -14° for 2 months. At about 2° the stock solution lost 2–5% activity in 7 days and 50% if diluted twenty times in 0.025M-potassium chloride. At 40° between pH 5.0 and 7.5 there was a loss of 2–3% after 40 min. In crude extracts the enzyme was much less stable at 40° , but various batches of cells and extracts showed considerable variation in stability; generally the crude extracts were more stable at pH 7.5 than at pH 5.0. At pH 9.0 enzyme activity of both crude and purified extracts was irreversibly lost in less than 10 sec., even at 2° . On the other hand, some of the enzyme activity lost when crude extracts were incubated without substrate at pH 4.0–5.0 was recovered when solutions were taken to pH 8.5 with phosphate buffer and re-tested at pH 5.0.

Table 4. *The effect of inhibitors on the rate of deamidation by purified glutaminase*

(To one side of a double side arm tube was added a solution of the inhibitor in 0.2M-acetate buffer, pH 5.0, 0.01M-glutamine. To the other side arm was added enzyme (0.004 mg. total N/0.5 ml.). Final volume 2.0 ml. After 1 min. at 40° the contents of the tube were mixed and rates of deamidation were determined as described.)

Inhibitor added	Concn. of inhibitor (M $\times 10^{-3}$)	Inhibition (%)
γ -Glutaminy l hydrazide	50	0
Methionine sulphoxide	20	0
'Atebrine'	10	0
'Euflavine'	10	0
CuSO ₄	{ 10 1.0	{ 85 10
Iodoacetate	{ 2.0 2.5	{ 85 50
NaF	2.5	50
NaNO ₂	2.0	35
NaN ₃	1.0	5.0
Na ₂ SeO ₄	2.5	80
NaHSO ₃	2.5	100
N ₂ H ₄ (H ₂ SO ₄)	1.0	0
NH ₂ OH(HCl)	1.0	5.0
Phenolphthalein	1.0	10
Phenol red	1.0	22
Thymol blue	1.0	40
Bromocresol purple	1.0	85
Bromocresol green	{ 1.0 0.2 0.05	{ 100 80 20
Bromsulphalein*	{ 1.0 0.2 0.05	{ 100 50 20

* Disodium sulphonate of phenoltetrabromophthalein.

The effect of inhibitors. Inhibitors of glutaminase at a concentration of about 10^{-3} M were selenite, sulphite and iodoacetate and certain phthalein dyes (Table 4). Two glutamine analogues, γ -glutaminy l hydrazide and methionine sulphoxide do not inhibit. The acridines tested and found inactive were shown by Archibald (1944) to inhibit the

glutaminase of animal tissues. This difference is possibly connected with the different pH at which the systems were tested (see Albert, Rubbo, Goldacre, Purvoy & Shoro, 1945).

The effect of bromocresol green (0.002M) on the initial rate of deamidation was measured at substrate concentrations from 0.033 to 0.00125M (Table 5). Inhibition was found to be dependent on substrate concentration and may thus be regarded as competitive. Similar results were obtained with bromsulphalein. Treatment of the results according to Lineweaver & Burk (1934) gave a straight line whose ordinate intercept was almost the same as that of the experiment in the absence of the dye.

Table 5. *The effect of bromocresol green on the rate of deamidation of glutamine at various substrate concentrations*

(Each double side arm test tube contained glutamine; 0.5 ml. purified glutaminase (0.004 mg. total N/0.5 ml.); 0.2M-acetate buffer, pH 5.0; 0.0025M-KCl; total vol. 2.0 ml. The reaction was stopped and NH₃ estimated as described.)

Glutamine concn. (M)	μ l. NH ₃ /5 min.	
	Without bromocresol green	With bromocresol green (2×10^{-4})
0.033	43.3	29.6
0.010	43.3	27.6
0.005	43.0	27.4
0.0025	40.0	16.9
0.002	37.9	14.8
0.0017	37.2	12.8
0.00125	34.9	7.6

Inhibition by bromocresol green increased as pH was lowered below the optimum. The pH curve of purified enzyme in the presence of the dye resembled that of the crude extracts shown in Fig. 1.

Bromocresol green also inhibited deamidation in intact cells and crude extracts. Glutamic acid decarboxylase in the cells was not inhibited by bromocresol green; thus glutamate could be estimated in the presence of glutamine by modifying the method of Gale (1947) and adding bromocresol green (0.03M) to the cells. Under these conditions the inhibition by bromocresol green was reversed by cetavlon, and this fact was used to devise a manometric method for estimating glutamate and glutamine in the same solution. Unfortunately different batches of cells varied widely in their behaviour towards both cetavlon and bromocresol green and the method was unsuitable for routine purposes. Similar results were given by bromsulphalein.

The effect of anions

The effect of chloride on partially purified glutaminase. Precipitates obtained by the addition of acetic acid to extracts of cells (e.g. stage 3 or 4,

Table 6. *The effect of salts on the restoration of activity to washed acid precipitates of glutaminase*

(Acid-precipitated glutaminase (F_2) was dissolved in 0.2M- Na_2HPO_4 , pH 8.3, precipitated by the addition of acetic acid and washed with water as described in the text. The washed precipitate was then dissolved in buffer and salt mixtures and the enzyme activity determined under the standard conditions.)

Buffer	Additional salt added	$\text{NH}_3/15 \text{ min.}$ (μL)
0.05M-Borate-KCl, pH 8.5	None	82
0.05M- Na_2HPO_4 , pH 8.3	None	8.8
0.05M- Na_2HPO_4 , pH 8.3	0.05M-Sodium borate	8.8
	0.05M-NaCl	82
	0.05M- KH_2PO_4	8.8
	0.05M-KCl	82
	0.015M-HCl	75
	0.01M- CaCl_2	73

Table 1) lost 75% of their glutaminase activity when washed with water and dissolved in phosphate buffer. On the other hand, there was little loss (<5%) if the washed precipitates were dissolved in borate-potassium chloride buffers, pH 8.5-9.5. The lost activity of the phosphate solutions was restored by the addition of various chlorides, but not by borates or many other salts of potassium or sodium (Table 6). The loss of activity upon washing can thus be explained by the removal of an activator which is replaced by chloride.

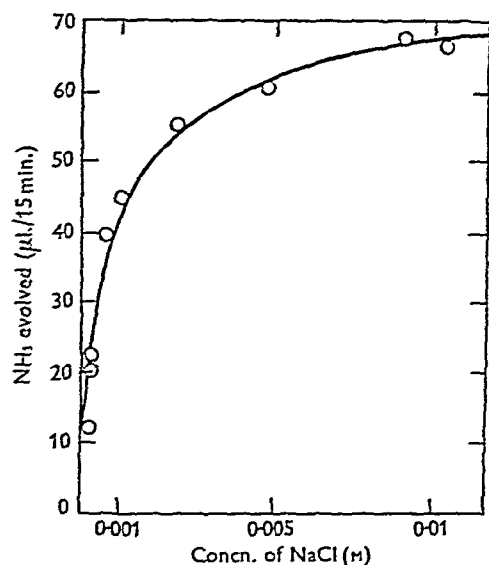


Fig. 4. Deamidation by glutaminase at varying concentrations of chloride. Each tube contained in a total volume of 2.0 ml.: 0.005M-glutamine; 0.2M-acetate buffer, pH 5.0; washed enzyme equiv. 0.25 mg. F_2 (see text) and varying concentrations of NaCl, where all concentrations are final concentrations.

To study the effects of various anions in detail a 'washed' enzyme was prepared by triturating 60 mg. powdered acid-precipitated glutaminase (F_2 ; Table 1) with 2 ml. of 0.05M- Na_2HPO_4 . A small amount of undissolved solid was removed by centrifuging and to the clear supernatant was added 4 ml. of 0.2M-acetate buffer, pH 4.0. After standing

at 2° for 20 min. the precipitated glutaminase was collected by centrifugation and washed five times with 5.0 ml. of ice-cold distilled water. The washed powder was redissolved in 2 ml. 0.05M- Na_2HPO_4 and diluted with water to 120 ml. This treatment reduced the activity by 80-85%.

Addition of chloride (0.025M) restored 95-100% of the lost activity (Table 6). The optimal concentration of chlorides was 0.02M (Figs. 4, 5). Equivalent amounts of sodium, magnesium, calcium, ferric, manganous and cobalt chlorides had similar effects. The effect of chloride was also demonstrated on samples of the stock purified glutaminase solution which had been dialysed against distilled water. Prolonged dialysis against water (beyond 2 hr.) resulted in irreversible loss of activity.

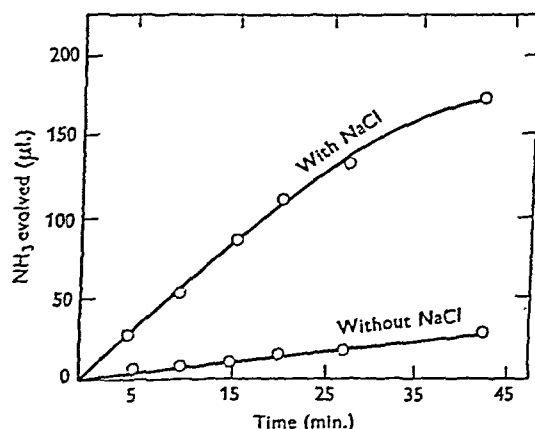


Fig. 5. The effect of chloride on the progress of deamidation. Each tube contained in a total volume of 2.0 ml.: 0.005M-glutamine; 0.2M-acetate buffer, pH 5.0; washed enzyme preparation equiv. 0.25 mg. F_2 (see text) and 0.025M-NaCl when added.

Effect of chloride on intact cells. One batch of cells of strain SR 12 was washed six times in distilled water and another five times in 0.02M-acetate buffer, pH 5.0; their chloride concentration fell from 0.03 to 0.01M and was not reduced by further washing. No enzyme activity was lost on washing and addition of chloride did not increase it. The

washed cells were ground with powdered glass, and extracted with acetate buffer (Hughes, 1949), and the extract diluted to contain the equivalent of 2.5 mg. dry weight cells/2.0 ml. The chloride concentration of the diluted extract was $2 \times 10^{-5} M$, part of the chloride having come from the glass. The addition of potassium chloride ($0.0025 M$) to this extract accelerated deamidation by 250 %.

Anions other than chloride and the rate of deamidation. In addition to chloride, some other monovalent anions in the following order of effectiveness: bromide > chloride > iodide > cyanide and thiocyanate > nitrate, accelerated the rate of deamidation by washed or dialysed preparations of glutaminase (Table 7). Sulphate, phosphate and arsenate

had no effect and nitrite, fluoride, sulphite, selenate and selenite inhibited.

The effect of chloride on the stability of glutaminase. Dialysed stock solutions of glutaminase lost 50–60 % of their enzyme activity when incubated for 20 min. at 40° . Only 3–5 % of the enzyme activity was lost when $0.025 M$ potassium chloride was added prior to incubation. Chloride thus protects the enzyme against inactivation by heat. About 6–10 % enzyme activity was lost upon incubating the dialysed solution with glutamine ($0.05 M$) for 20 min. showing that the substrate also protects the enzyme against inactivation. The acceleration of deamidation by chloride is therefore not due wholly to its protecting the enzyme against inactivation and it is likely that it plays some role in the catalysis of the reaction. Similar effects have been described for nickel, chromium and manganese in activating and stabilizing arginase (Stock, Hellerman & Perkins, 1938).

Table 7. *The effect of anions on the rate of deamidation of glutamine by washed glutaminase*

(Each tube contained: in 2 ml. solution $0.005 M$ glutamine; $0.2 M$ acetate buffer, pH 5.0; washed enzyme solution equiv. 0.25 mg. washed F_3 (see text). All concentrations are final concentrations.)

Salt added	Concentration ($M \times 10^{-3}$)	NH_3 formed in 15 min. ($\mu l.$)
None	—	9.0
KBr or NaBr	25	90
	10	77
	1.0	53.5
	0.1	19.5
KCl or NaCl	25	81.5
	10	72
	1.0	38.5
	0.1	16.5
KI or NaI	25	77
	10	71
	1.0	42
	0.1	22
KCNS or KCN	25	61
	10	61
	1.0	22.5
	0.1	12
$NaNO_3$	25	59
	10	51
	1.0	22
	0.1	11

The effect of cetavlon

The acceleration of deamidation by cetavlon in intact cells. The glutaminase activity and the action of cetavlon upon it was found to be dependent on the length of the growth period. Washed suspensions of strains SR 12 and 1490 grown on the laboratory medium or on papain digest or on semi-synthetic media had a low glutaminase activity until growth reached the middle of the log phase (2–3 hr. after inoculation); enzyme activity then increased rapidly. Growth ceased at 5–5.5 hr. after inoculation, but the cells continued to form glutaminase and maximal enzyme activity occurred 1.5–2 hr. after growth had ceased. No acceleration of deamidation by cetavlon was found in young cells; two- to threefold acceleration occurred after cessation of growth (Table 8). Maintaining the pH of the medium at 6.8–7.0 by the addition of alkali during growth or the addition of glutamine ($0.01 M$) has no effect on the behaviour of glutaminase.

Acceleration of deamidation by cetavlon in extracts. In extracts of cells grown for 16 hr. on the laboratory

Table 8. *Age of culture and glutaminase activity of Clostridium welchii (strain 1490)*

(Cells were grown at 37° on casein-meat-glucose medium, washed twice with 0.9 % NaCl and suspended in $0.05 M$ acetate buffer, pH 5.0 (4–20 mg./ml.). The glutaminase reaction and estimation of ammonia were carried out as described in the text. Dry weight of cells estimated turbidometrically and pH with a glass electrode.)

Time (hr.)	Dry wt. cells (mg./ml. medium)	pH of medium after growth	$q_{NH_3}^N$	
			Without cetavlon	With cetavlon ($1.25 \times 10^{-3} M$)
0	—	6.8	—	—
2.5	0.13	6.3	360	360
4	0.37	5.1	850	850
5.5	0.43	4.8	1120	1540
7	0.43	4.7	1560	2120
13.5	0.40	4.7	2560	4250

Table 9. *The effect of cetavlon on deamidation by various glutaminase preparations*

(Each tube contained: 0.005M-glutamine; 0.2M-acetate buffer, pH 5.0; 0.025M-KCl; enzyme solution as described below. Final vol. 2.0 ml. Temp. 40°.)

Enzyme preparation	pH	NH ₃ (μ l./10 min.)	
		Without cetavlon	With cetavlon (5×10^{-4} M)
A, 16 hr. culture of strain SR 12 (2.5 mg./2.0 ml. final)	5.0	90	210
	4.0	66	140
B, extract prepared from the above cells after drying <i>in vacuo</i> (equiv. 2.5 mg./2.0 ml. final)	5.0	90	90
	4.0	60	90
C, extract of above cells broken with powdered glass (equiv. 3.0 mg./2 ml. final)	5.0	98	110
	4.0	30	100
D, above extract made to pH 4.0 with acetic acid. Precipitate removed by centrifuging and suspended in 0.05M-acetate buffer, pH 5.0	5.0	85	85
	4.0	30	80
E, precipitate at pH 4.0 in the above experiment. Dissolved in 0.2M-Na ₂ HPO ₄ and treated with safranin and Zeo-Karb (0.003 mg. total N/2.0 ml.)	5.0	45	22
	4.0	40	35

medium and obtained by crushing with powdered glass, deamidation was accelerated 20–30 % at pH 5.0 and 300–700 % at pH 4.0. Thus the accelerating effect of cetavlon in extracts was about one-fifth of that in intact cells at pH 5.0 and two to three

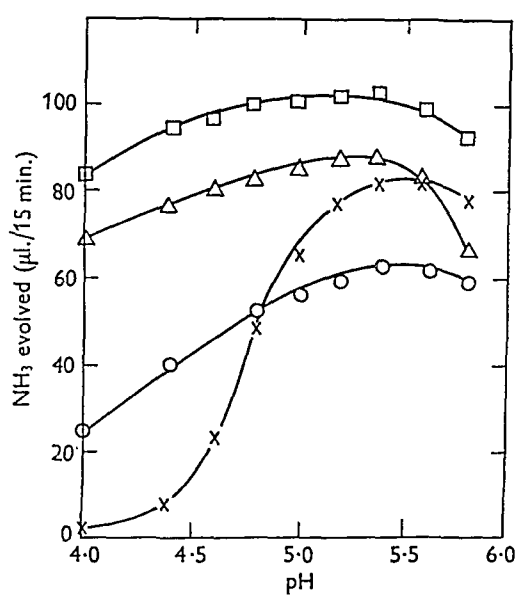


Fig. 6. The effect of cetavlon on the pH curve of glutaminase in intact cells and extract. *Cl. welchii* SR 12 was grown for 16 hr. on the laboratory medium, and the extract prepared by grinding with powdered glass as previously described (Hughes, 1949). Glutaminase activity was estimated as described in the text. ○—○, intact cells; □—□, intact cells with cetavlon; ×—×, extract; △—△, extract with cetavlon.

times greater at pH 4.0. In extracts of dried cells the effect of cetavlon was generally less marked, but showed the same increase at the lower pH values (Table 9). Fig. 6 shows that cetavlon changed the

pH curve of the enzyme in intact cells and extracts by broadening the optimum towards the lower pH range. Differences in the effect of cetavlon between the various enzyme preparations could thus be partly accounted for by differences in their pH curve, i.e. the steeper the pH curve in the acid range the greater was the effect of cetavlon.

Acceleration by cetavlon decreased as the extracted enzyme was purified and, as shown later, deamidation in the finally purified enzyme was inhibited by the usual concentration of cetavlon (2.5×10^{-3} M). In the course of purification of the enzyme, the accelerating effect remained unchanged after one precipitation of the enzyme with acetic acid at pH 4.0. Some diminution occurred on repeated precipitation and solution in 0.2M-borate buffer, pH 8.5 (Exp. D, Table 9). Treatment of the enzyme solutions with safranin resulted in the complete loss of accelerating effect (Exp. E, Table 9). At this stage the pH curve of the enzyme was the same as that in the finally purified solutions. In these experiments the cetavlon concentration was reduced to 5×10^{-4} M in order to avoid inhibiting effects at the later stage of purification. This concentration gives the maximum acceleration (Hughes 1950) in cells and crude extracts.

The effect of cetavlon at varying substrate concentrations. In intact cells and in crude extracts cetavlon increased the rate of deamidation at substrate concentrations below 0.04M, but not at 0.1M (Table 10). The substrate concentration-activity relation, plotted according to Lineweaver & Burk (1934), did not give straight lines possibly because diffusion is one of the limiting factors. The concentration at which the velocity was half maximal was decreased from 0.025 to 0.013M by 2.5×10^{-3} M-cetavlon. Cetavlon thus increased the apparent affinity of glutaminase for glutamine.

Table 10. *The effect of cetavlon on the rate of deamidation of glutamine at varying substrate concentrations*

(Experimental details were as described in Table 3, but intact cells (2.5 mg. dry wt./ml.) were used in the place of purified extract.)

Concentration of glutamine (M)	NH ₃ formed (μl.)	
	Without cetavlon	With cetavlon (1.25 × 10 ⁻³ M)
0.100	148	151
0.035	109	126
0.013	79	110
0.010	62	111
0.004	47	84
0.002	34	61

Inhibition of deamidation by cetavlon. As already mentioned, relatively low concentrations of cetavlon inhibited deamidation in the purified extract (Exp. A, Table 11) and had little effect in intact cells and crude extracts (Exps. B and C, Table 11).

Table 11. *Inhibitory effect of cetavlon*

(Each tube contained: 0.005 M-glutamine; 0.2 M-acetate buffer, pH 5.0; 0.025 M-KCl. Enzyme solution as described below. Total vol. 2.0 ml. Temp. 40°.)

Enzyme preparation	Cetavlon concentration (× 10 ⁻³ M)	NH ₃ (μl./10 min.)
A, purified glutaminase (0.004 mg. total N)	0	106
	0.002	105
	0.08	90
	0.13	90
	1.25	70
	2.5	47
B, <i>Cl. welchii</i> strain SR12 grown for 2.5 hr. (0.25 mg. total N)	5.0	35
	0	90
	0.8	101
	1.25	108
	2.5	110
	5.0	84
C, extract of the above cells after grinding with powdered glass (0.35 mg. total N)	0	90
	0.008	93
	0.3	82
	1.25	93
	2.5	90
	5.0	81

Table 12. *The effect of pH on the inhibition of deamidation by cetavlon*

(Each tube contained: 0.005 M-glutamine; 0.2 M-acetate buffer, pH 5.0; 0.025 M-KCl; purified enzyme solution (0.004 mg. total N). Total vol. 2.0 ml. Temp. 40°.)

pH of reaction	NH ₃ (μl./10 min.)		Inhibition (%)
	Without cetavlon	With cetavlon (0.0025 M)	
5.5	94	55	42
5.0	108	54	50
4.7	105	84	20
4.5	101	89	11
4.3	101	90	11
4.0	96	92	4
3.6	85	81	5

This inhibition decreased at pH values below the optimum and was negligible at pH 4.0 with the usual concentration (2.5 × 10⁻³ M) of cetavlon (Table 12). At this pH there was no acceleration of deamidation when the concentration of detergent was reduced to 5 × 10⁻⁴ M.

DISCUSSION

The present experiments upon the acceleration by cetavlon of the deamidation of glutamine in intact cells and crude extracts of *Cl. welchii* confirm the previous suggestion (Hughes, 1949) that the detergent increases the affinity of the glutaminase for its substrate. As judged by the apparent Michaelis constants (Table 13), increase in affinity also resulted from purification of the enzyme. Parallel

Table 13. *The apparent Michaelis constants of various glutaminase preparations*

(The results of measuring the rate of deamidation at various substrate concentrations were plotted in the form of initial velocity against substrate concentration. Maximal velocity was estimated by extrapolation and the figures in the table are the concentration of substrate at half maximal velocity.)

Enzyme preparation	pH	Apparent Michaelis constant
Intact cells of strain SR12	5.0	0.005
Above cells with cetavlon (0.0025 M)	5.0	0.002
Extract of the above cells as described by Hughes (1949)	4.1	0.012
Above extract with cetavlon (0.0025 M)	4.1	0.0025
Purified extract*	5.0	0.00063–0.00060
Purified extract*	4.1	0.00070

* These results agreed with those obtained by plotting according to Lineweaver & Burk (1934).

with the increase in affinity during purification the accelerating effect of cetavlon decreased, and in the purified extracts the detergent did not accelerate, but, under some conditions, inhibited deamidation. It is unlikely, therefore, that the increase in the affinity of the enzyme for substrate is due to a reaction between cetavlon and the glutaminase, since if this were so, acceleration would be found in purified as well as crude enzyme preparations. The previous suggestion (Hughes, 1949), that the detergent accelerates the enzyme reaction by removing a competitive inhibitor, therefore seems eminently feasible. Mechanisms by which an inhibitor might be removed by cetavlon have already been discussed (Hughes, 1950) and the presence of detergent micelles shown to be important.

An additional inhibitory effect, not reversible by cetavlon, was noticed in activity measurements on crude enzyme preparations at varying substrate concentrations. Treatment of the results according

to Lineweaver & Burk (1934) suggested that diffusion limited the rate of deamidation at the lower substrate concentrations.

The acceleration of deamidation by anions. The activation of washed and dialysed glutaminase by monovalent anions is especially interesting since, so far as the authors are aware, this is the first occasion in which activation of a bacterial enzyme by univalent anions has been reported. The order of effectiveness of the anions activating bacterial glutaminase is similar to that of the anions activating salivary and pancreatic amylase (Hoffmeister, 1888; Pringsheim, 1912). No bacterial amylase so far studied is activated by anions (Hockenhull & Herbert, 1945; Kneene & Beckford, 1946; Di Carlo & Redfern, 1947). Renal glutaminase and the glutaminase of rat liver has been shown to be activated by sulphate, arsenate and phosphate (Mylon & Hellor, 1948; Greenstein & Leuthardt, 1948; Errara, 1949; Errara & Greenstein, 1949). Neither these anions nor chloride and bromide accelerate the non-enzymic hydrolysis of glutamine (Gilbert, Price & Greenstein, 1949). The present experiments do not make it possible to suggest a mechanism for the acceleration of deamidation by chloride and other anions, but it seems likely that they play some role in catalysing deamidation as well as in protecting the glutaminase against heat inactivation.

Comparison between the glutaminase of Cl. welchii and those from other sources. The glutaminases of *Proteus morganii* (McIlwain, 1948) and *Escherichia coli* (Hughes, 1949) have approximately the same optimum pH as that in *Cl. welchii*, but experiments not reported here suggest that they are not accelerated by chloride. In streptococci (McIlwain, Roper & Hughes, 1948) the breakdown of glutamine occurs only in the presence of a concomitant reaction with glucose and is inhibited by γ -glutaminy hydrazide. Glucose does not affect the rate of deamidation in *Cl. welchii* and γ -glutaminy hydrazide is also without effect. The main similarities between the glutaminases from animal tissues and that from *Cl. welchii* are that they are hydrolytic enzymes which yield ammonia and glutamic

acid quantitatively without the formation of pyroglutamate and are inhibited by bromosulphalein. The main differences in the enzymes from the two sources are that the optimum pH of the enzymes in animal tissues is from 8.0 to 9.0 (Archibald, 1945), while that in *Cl. welchii* is at 5.0; and as already mentioned above there is a difference in the anions which activate the enzymes.

SUMMARY

1. The glutaminase of *Clostridium welchii* strains SR 12 and 1490 has been extracted from dried cells and purified from 40- to 60-fold in different batches, by fractionation between pH 4 and 5 and treatment with safranin.
2. The affinity of the enzyme for its substrate, its efficiency below the optimum pH, and its solubility, increased upon purification.
3. Cetavlon accelerated deamidation by intact cells reaped towards the end of the lag phase of growth, but not by younger cells.
4. The accelerating effect of cetavlon in intact cells and crude extracts was due to an increase in the affinity of the enzyme for its substrate. In purified extracts cetavlon did not accelerate, but inhibited, deamidation. These results suggest that the acceleration is due to the removal of a competitive inhibitor.
5. The partially purified glutaminase lost activity (75 %) upon washing with or dialysis against water. The lost activity was restored by some univalent anions in the following order of effectiveness: bromide > chloride > iodide > cyanide > thiocyanate and nitrate.
6. Inhibitors of glutaminase included phenolphthaleins and sulphophthaleins. The action of one of these, bromocresol green, has been shown to be competitive. The dye also alters the shape of the pH curve of the enzyme by decreasing its efficiency below the optimum pH.

The authors wish to express their thanks to Prof. H. A. Krebs, F.R.S., for his help and advice. They are also grateful to Dr B. C. J. G. Knight for his gifts of two large batches of cells of *Cl. welchii*.

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The Quantitative Determination of Dehydrogenase Activity in Cell Suspensions

By A. R. FAHMY AND E. O'F. WALSH

Department of Physiology, Royal Free Hospital School of Medicine, London

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While investigating the action of nicotine on the enzyme systems of yeasts we attempted to use variations of the classical Thunberg technique to compare the behaviour of the dehydrogenases of a normal yeast with those of one which had been grown in the presence of nicotine. The methods proved unsuitable with the apparatus available because the suspended cells prevented reliable measurement of the residual colour with an ordinary photoelectric absorptiometer and some of the methylene blue was always firmly adsorbed on to the cells. If, on the other hand, the time required for complete decolorization be measured, it is difficult to draw conclusions from comparative inhibitions or activations because the concentration of the hydrogen acceptor becomes a limiting factor during the course of an experiment.

Triphenyltetrazolium chloride (TTC), which yields a water-insoluble red formazan on reduction (Kuhn & Jerschel, 1941; Straus, Cheronis & Straus, 1948), has advantages over methylene blue as hydrogen acceptor in that it can be present in excess throughout an experiment and it can be used in the presence of air. The colorimetric method of Kun & Abood (1949), which employs TTC for the determination of succinic dehydrogenase activity in mammalian tissue homogenates, depends on the solubility of the formazan in acetone. Their method cannot be applied to yeast suspensions since acetone fails to extract the colour from the cells. Even with mammalian tissues they found it necessary to prepare reference standards in order to correct for the unequal adsorptions of the dye by different tissues and Becker (1949), who investigated the suitability of TTC as a histochemical stain for dehydrogenase, observed that the formazan was deposited in and around the tissues in an irregular manner.

In the present paper a method is described which is suitable for the quantitative determination of the activity of various dehydrogenases in yeast suspensions.

EXPERIMENTAL

Colorimetry. All measurements were made with a Hilger Biochemical absorptiometer using a blue filter, OB2.

Yeast suspension. Except where otherwise stated, a suspension in distilled water of the washed cells of *Saccharomyces cerevisiae* was aerated for 2 hr. at 37°, centrifuged and the cells were resuspended in distilled water.

Triphenyltetrazolium bromide (TTB). A 0.5% (w/v) solution in distilled water of the commercial preparation 'Grodex' (May and Baker Ltd.) was freshly prepared or stored in a refrigerator.

Phosphate buffer. Clark & Lubs's standard buffer, 0.05M and pH 7.6.

Other chemicals. All materials used were of ordinary reagent purity.

RESULTS

In the preliminary experiments a suspension (1.0 ml.) of washed, but not aerated, yeast cells was mixed with phosphate buffer, pH 7.6 (2.5 ml.) and TTB solution (0.5 ml.) and the mixture was incubated at 37° until a distinct red colour had developed. Mixtures, so prepared, were mixed either with acetone (1–2.5 vol.) as in the method of Kun & Abood (1949) or with ethanol (1–2 vol.); or they were shaken with either toluene, xylene, chloroform, isobutanol or amyl alcohol. Similar attempts to extract the colour were made with mixed solvents (e.g. toluene with methanol or pyridine) and with the mixture rendered either acid or alkaline by the addition of sulphuric acid or sodium hydroxide. None of these methods was successful.

If the tubes containing the mixture with ethanol (1.5 vol.) were immersed in boiling water for 3 min., the colour was extracted completely from the cells,

but such a method has obvious disadvantages. Addition of glacial acetic acid (1.5 vol.) to the mixture dissolved the formozan which could then be extracted readily and completely by shaking with toluene. With smaller proportions of acetic acid the dye can be extracted from the cells, but stable emulsions result and it is difficult to separate the toluene layer.

Colorimetry of toluene solutions

Solutions in toluene of the formozan in known concentrations were prepared by extraction of solutions which contained measured quantities of TTB and to which a few crystals of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) had been added. The linear relation-

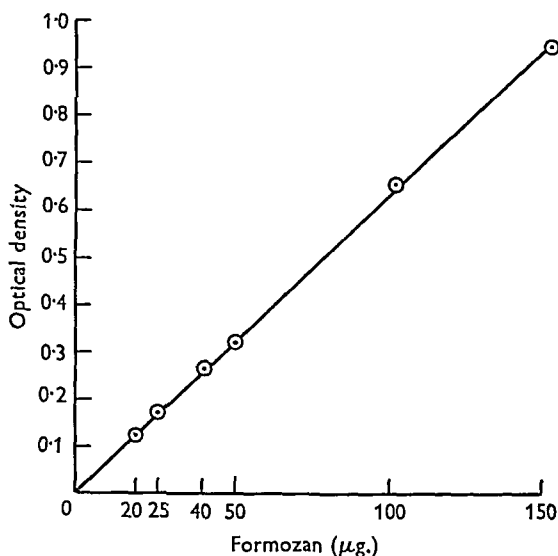


Fig. 1. Relationship between optical density and concentration of formozan (reduced triphenyltetrazolium bromide) in toluene. For details see text.

ship between optical density and the concentration of the dye in toluene is shown in Fig. 1.

Solutions of the formozan in either toluene or xylene are clear red, and the colour is stable for several hours, whereas the colour of solutions in the oxygen-containing solvents (alcohols, acetone, acetic acid) is dull orange-red and is somewhat less stable.

Measurement of dehydrogenase activity

Reaction mixtures (4 ml.) containing the cell suspension, buffer, substrate, TTB solution (0.5 ml.) and any other reagents are prepared in glass-stoppered tubes. Controls without substrate are also prepared. The mixtures are incubated at the desired temperature and, after a suitable interval, reaction is arrested by the addition of acetic acid

(6 ml.). The acidified mixtures are shaken with toluene (6–10 ml.), centrifuged if necessary, and the coloured toluene layers are compared in a photoelectric absorptiometer.

Effect of enzyme concentration

The colours developed in a series of mixtures, prepared as above, but which contained different amounts of non-aerated yeast cell suspension, were compared. The results, which demonstrate the linear relationship between the optical density of the toluene extracts and the concentration of the mixed dehydrogenases, are shown in Fig. 2. Similar results have been obtained with aerated yeast suspensions and either glucose or ethanol as substrate.

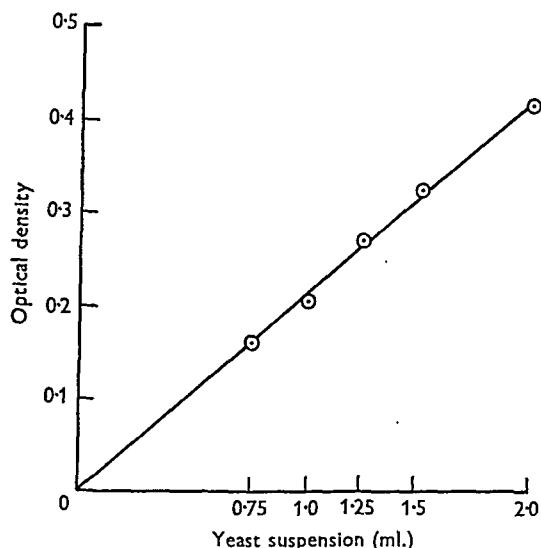


Fig. 2. Relationship between colour developed and quantity of yeast suspension. For details see text.

Microscopical examination of the cells remaining after an experiment confirmed that none of the colour had remained with the cells.

By means of the procedure, here described, we have been able to obtain consistent results on measuring the effects of nicotine on the activity of various dehydrogenases in living yeast cells. Consistent results were not obtained when we used other, previously available, methods.

SUMMARY

A colorimetric method is described for the quantitative determination of dehydrogenase activity in yeast and other cell suspensions. The method is especially useful when it is desired to measure the effects of either activators or inhibitors on dehydrogenase systems.

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Adaptation of *Bacillus subtilis* to Fatty Acids

By H. LASER

Molteno Institute, University of Cambridge

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It has previously been shown (Laser, 1951) that respiration and growth of *Bacillus subtilis* are inhibited by *cis*-vaccenic acid, the degree of inhibition being dependent on the ratio of acid to cells. The inhibition is transient at suitable ratios, respiration and growth ultimately proceeding at rates equal to or even greater than those of untreated controls. Bacterial cells, which have recovered from the initial inhibition by vaccenic acid, were found to be resistant to further addition of the acid, a result which resembles acquired drug resistance by bacteria. These studies have now been extended to other fatty acids and the behaviour of such treated cells after subculture in absence of fatty acids has been examined.

MATERIAL AND METHODS

The strain of *B. subtilis* used was no. 2586 of the National Collection of Type Cultures. Vegetative forms were obtained and prepared for manometric experiments as previously described (Laser, 1951).

The medium in which washed cells were suspended was 0.15M-phosphate buffer, pH 8.0, containing 2% glucose \pm 0.5 ml. nutrient broth/3.0 ml.

The fatty acids tested were stearic, linoleic, oleic and synthetic *cis*-vaccenic acid (Morton & Todd, 1950). They were dissolved with heating in 0.3M- K_3PO_4 (usually 2.0 mg./ml.), diluted with water to 0.15M, and pipetted into the reaction vessel immediately before adding the organisms. The acids and their solutions in K_3PO_4 were kept under N_2 in the cold. The solutions were warmed before use and in the case of stearic acid, all the components of the medium were heated to 40° and quickly pipetted into warmed vessels. In order to maintain the pH of the phosphate buffer at pH 8.0, 0.12 ml. 0.15M- KH_2PO_4 were added per 0.1 ml. 0.15M- K_3PO_4 containing the dissolved fatty acid.

Respiration was measured manometrically in Barcroft-Warburg manometers at 37°. The gas phase was O_2 . As a rule all components of the medium were filled into the main compartment of the flasks before the beginning of the experiment, the fatty acid being added immediately before the organisms. In those experiments in which the fatty acid was added at $t = 0$ or at a convenient time during the course of the experiment, it was tipped into the main compartment of the vessel from a side bulb, the required amount of acid being dissolved in 0.05 ml. 0.15M- K_3PO_4 . The same amount of 0.15M- K_3PO_4 was tipped at the same time into the control vessel.

The initial (dry) weight of cells per vessel varied between 0.5 mg. (growing cells) and 1.0–2.0 mg. (washed cells), contained in 0.1–0.4 ml. of a suitably diluted washed

suspension. Its turbidity was determined by means of the Spekker absorptiometer (1 cm. cell, Ilford filters H 503 and 608) and the cell content (on a dry weight basis) obtained by reference to a calibration curve.

To subculture cells after treatment with a fatty acid at the end of a manometric experiment, the following procedure was observed. One small drop of the cell suspension was plated on nutrient agar in a Petri dish and incubated overnight at 37°. In order to exclude accidental contamination during the manometric procedure, the culture was examined microscopically and cells from a single colony were then incubated in 10.0 ml. nutrient broth + 2% glucose for 8 hr. and transferred into Roux flasks upon peptone-agar for about 16 hr., as previously described (Laser, 1951).

Symbols and abbreviations. R = 'acid ratio' = ratio of wt. of fatty acid in the medium : dry wt. of cells. Recovered cells = cells whose respiratory and growth rates, after initial inhibition by a fatty acid, had equalled those of the untreated control. Q_{O_2} = μ l. O_2 uptake/mg. dry wt./hr.

RESULTS

The present experiments deal mainly with the effect of fatty acids on the respiratory rate of washed cell suspensions in phosphate buffer solution + glucose and on the respiratory and growth rates of cells in the same medium to which nutrient broth had been added. No growth occurs in washed suspensions in absence of broth, so that the rate of O_2 uptake, in absence or presence of fatty acid, is a measure of the ability to oxidize glucose. In growing cells the rate of O_2 uptake is, in addition, an index of growth, i.e. the logarithmic increase in cell population is reflected in the logarithmic increase of the respiratory rate.

The effect of stearic acid. Fig. 1 shows the effect of different concentrations of acid on the O_2 uptake of 2.0 mg. washed cells. It will be seen that (1) at the concentrations used, the respiration is initially inhibited; (2) the inhibition increases with increasing amounts of acid; (3) the cells gradually recover from the inhibitory effect of the acid and eventually attain a rate of O_2 uptake which approaches or equals that of the control. Similar curves are obtained if the same concentration of acid is allowed to act on decreasing amounts of cells, i.e. the inhibition becomes greater as the cell concentration falls. This confirms earlier results with vaccenic acid (Laser, 1951) and demonstrates that the inhibition depends much less on the concentration of acid in the medium than on the ratio of acid to cells. This ratio (R) has

therefore been used throughout the descriptive part of the paper while the corresponding molar concentrations are given in the figures.

Stearic acid, which was the only saturated acid tested, has been found to inhibit respiration less than the unsaturated acids. This became especially apparent at higher concentrations, the cells tolerating and recovering from acid ratios as high as 1, which was not the case with the other acids. Such a result could be ascribed to the low solubility of stearic acid which might prevent a uniform distribution of acid among the cells. Preliminary experiments, however, in which the precaution of warming

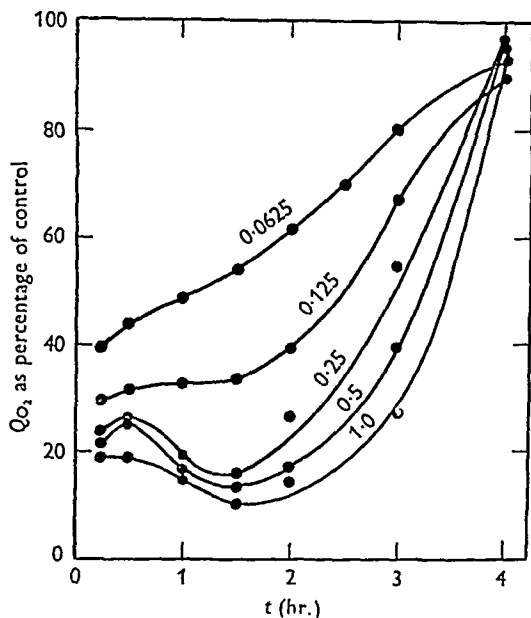


Fig. 1. Effect of varying ratios of stearic acid: cells (R) on O_2 uptake of washed suspensions (2.0 mg. dry wt./4.7 ml.), plotted as % of Q_{O_2} (μ l. O_2 /mg. dry wt./hr.) of control in absence of acid; 0.15M-phosphate buffer, pH 8.0, +2% glucose; gas phase, O_2 . The figures on the curves denote R . Concentration of acid in the medium (from top to bottom): 0.94 , 1.89 , 3.75 , 7.57 and $15.1 \times 10^{-4}M$.

all components of the medium prior to setting up the vessels was not taken (see Methods), allowed this possibility to be excluded. Under these conditions the paradoxical effect was obtained that the inhibition of O_2 uptake increased with decreasing concentrations of acid. This can be explained by the readiness of dissolved stearic acid to precipitate out of solution more quickly at high than at low concentrations, thereby diminishing the effective R . This effect was not encountered when the proper precautions were taken. The relatively low inhibitory effect of stearic as compared with the unsaturated acids therefore appears to be real. A similar observation, namely the relatively low toxicity of stearic acid as compared with unsaturated acids, has been made by Kodicek &

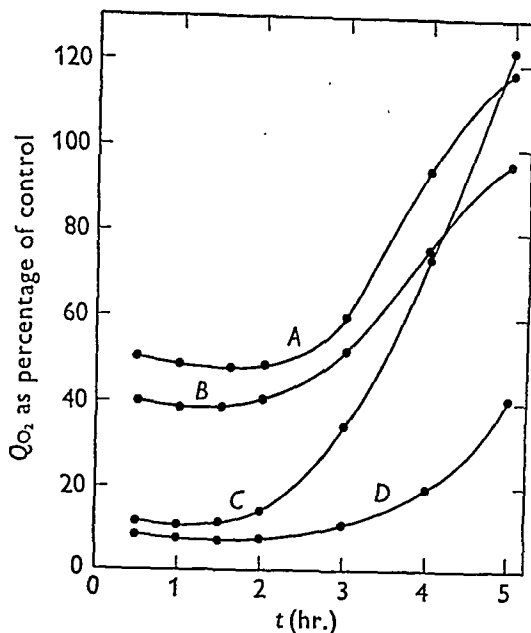


Fig. 2. Effect of different acids on O_2 uptake of washed cell suspensions (2.0 mg. dry wt./3.0 ml.), plotted as percentage of Q_{O_2} of control in absence of acid; 0.15M-phosphate buffer, pH 8.0, +2% glucose; gas phase, O_2 ; ratio of acid: cells = 0.05; concentration of acids in medium = $1.18 \times 10^{-4}M$; A, stearic; B, oleic; C, vaccenic; D, linoleic acid.

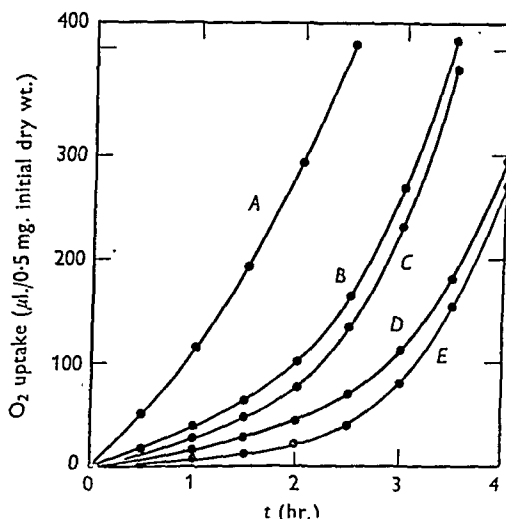


Fig. 3. Effect of different acids on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15M-phosphate buffer, pH 8.0, +2% glucose + 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 ; ratio of acid: cells = 0.05; concentration of acid in medium = $1.18 \times 10^{-4}M$; A, control without acid; B, stearic; C, oleic; D, vaccenic; E, linoleic acid.

Worden (1945) in their work on growth and lactic acid formation by *Lactobacillus casei*.

The relative effect of different fatty acids. Figs. 2 and 3, which demonstrate the effect of different

acids on the O_2 uptake of the micro-organisms at equal ratios of acid : cells, show that the magnitude of the initial inhibition increases in the following order:

stearic < oleic < vaccenic < linoleic.

Thus washed cells treated with linoleic acid, $R = 0.1$, did not recover from the respiratory inhibition during an experiment of 6 hr. duration, while with oleic acid at the same ratio recovery set in after 3 hr., leading after 6 hr. to a 70% increase of the

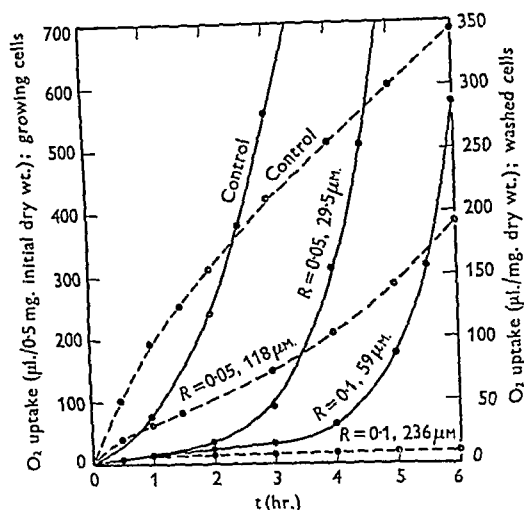


Fig. 4. Effect of varying ratios of linoleic acid : cells (R) on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.) and of washed suspensions (2.0 mg. dry wt./3.0 ml.); 0.15M-phosphate buffer, pH 8.0, + 2% glucose ± 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 ; growing cells, —●—; washed suspensions, - - -●- - -.

oxidation rate above that of the control. The inhibition of O_2 uptake of growing cells was always accompanied by an inhibition of growth, as in the case of vaccenic acid (Laser, 1951). When the cells recover from the respiratory inhibition, however, the rate of growth approaches or even exceeds the normal rate.

Relative tolerance for linoleic acid of washed and growing cells. The question of the necessity of growth for the development of resistance is an important one. The behaviour of washed and growing cells of the same batch was therefore examined on addition of two different concentrations of linoleic acid ($R = 0.05$ and 0.1) the higher of which inhibited the respiration of washed cells completely and without recovery (Fig. 4). It will be noted that growing cells are able to overcome the inhibition. The increased tolerance, however, conferred by the conditions of growth is small, only twofold, as at a ratio $R = 0.2$ no recovery took place even with growing cells, the corresponding curve (not shown in Fig. 4)

being superimposable on that representing washed cells at $R = 0.1$.

Repeated addition of fatty acids. Growing *B. subtilis*, which have been treated with and have recovered from the initial inhibition by vaccenic acid, are resistant to a further addition of this acid (Laser, 1951). It was therefore determined whether this is also the case with other fatty acids and whether the developed resistance is specific for the acid with which the cells have been treated. Fig. 5 shows the effect of a second addition of different amounts of linoleic acid on the respiration of washed cells, which are recovering from an initial inhibition

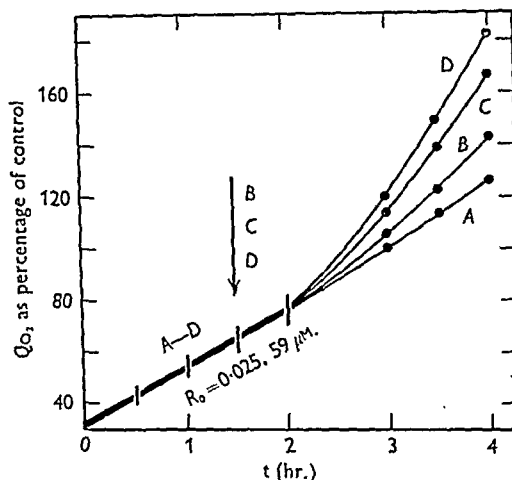


Fig. 5. Effect of second addition of linoleic acid on O_2 uptake of washed cell suspensions (2.0 mg./3.0 ml.) plotted as percentage of Q_{O_2} of control in absence of acid; 0.15M-phosphate buffer, pH 8.0, + 2% glucose; gas phase, O_2 . Initial ratio of acid : cells (R_0) and concentration of acid in the medium are shown on the heavy line (A-D), which indicates four initially identical vessels, to three of which (B, C, D) a second addition of acid (0.05, 0.1 and 0.2 mg.) has been made at $t = 1.5$ hr. (indicated by arrow), leading to $R_1 = 0.05$ for B, 0.075 for C and 0.125 for D.

by linoleic acid ($R = 0.025$). It will be seen that the cells not only tolerate amounts of acid in excess of the initial concentration, but show an increase of O_2 uptake with increasing amounts of added acid. This cannot have been caused by the oxidation of the acid itself, because it was many times greater than could be accounted for by the oxidation of the added acid. This increased rate of metabolism may be likened to, or be one of the underlying reasons for, the increased growth rate of certain bacteria, e.g. *Lb. casei*, in presence of oleic acid, after recovery from the primary growth inhibition (Williams & Fieger, 1946). Fig. 6 illustrates the effect of a second addition of acid to growing cells. Here too the treated cells, during the process of recovery, have become resistant to approximately six times the

initial acid ratio, as expressed by the steady logarithmic increase of respiration, which is a measure of the increase in cell population. In a number of experiments with oleic acid this has also been directly confirmed by density determinations. Similarly, treated recovered cells grew at a normal rate when transferred into a new medium in presence of fatty acid at a concentration several times higher than the initially inhibiting dose. Increasing the amount of acid by a second addition, however, did not produce an increase in the growth rate above that of the controls. This is probably due to the fact that, under the experimental conditions, growth proceeded at the maximal rate.

Enzyme adaptation by bacteria, however, is believed to occur fairly rapidly under suitable conditions. Thus Pollock (1950) has demonstrated that, after a brief treatment of bacteria with penicillin, an adaptive enzyme is produced within about 1 hr. In the experiments illustrated in Figs. 5 and 6 of this paper, a second addition of acid had therefore been made at a time when the cells were only beginning to recover from the respiratory inhibition. It will be seen that at that time resistance had already developed. It was then determined whether under the conditions of these experiments, which differ from those maintained by Pollock, a relatively

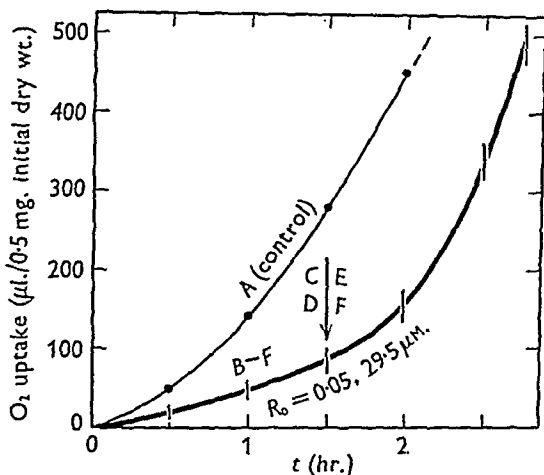


Fig. 6. Effect of second addition of linoleic acid on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15M-phosphate buffer, pH 8.0, +2% glucose +0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 . Initial ratio of acid to cells (R_0) and concentration of acid in the medium are indicated on the heavy line (B-F). This represents five initially identical vessels, to four of which (C-F) a second addition of acid (0.025, 0.05, 0.1 and 0.2 mg.) has been made at $t=1.5$ hr. (indicated by arrow), leading to approximate $R_t=0.066$ for C, 0.1 for D, 0.166 for E and 0.3 for F, on taking the increase in cell population at $t=1.5$ hr. into account.

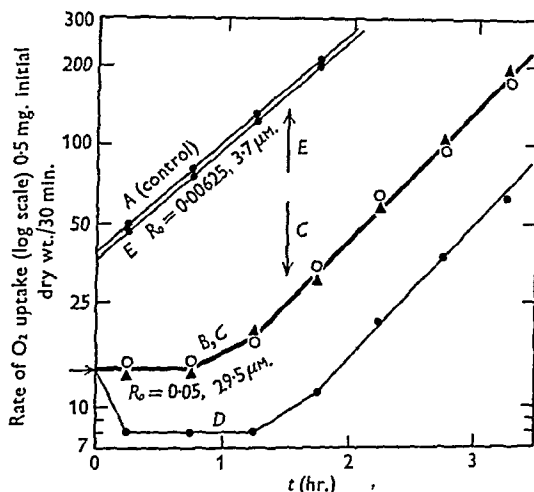


Fig. 7. Effect of second addition of oleic acid, at different times of the experiment, on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15M-phosphate buffer, pH 8.0, +2% glucose +0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 . Initial ratio of acid : cells (R_0) and concentration of acid in the medium are indicated in the figures on the curves (for curve D, see text). The heavy line (B, C) represents two initially identical vessels. Second additions (0.05 mg.) were made to C and E at $t=1.5$ hr., to D at $t=0$, leading to R_t (approximate R_t for C and E) = 0.0835 for C, 0.1 for D and 0.07 for E, on taking the increase of cell population at $t=1.5$ hr. into account.

The question whether the resistance is specific for the acid with which the cells have initially been treated was examined by cross-reaction of growing cells with all the acids used in these experiments. The results were uniform and unambiguous: treatment with any one acid produced resistance against any of the other acids. Even cells treated with and recovered from the inhibition by a saturated acid (stearic acid) showed unaffected rates of O_2 uptake and growth on addition of linoleic acid.

Development of resistance. In previous work (Laser, 1951) a second addition of acid to treated cells had been made at a later stage of the experiments, i.e. after complete recovery had taken place.

short contact of the bacteria with a fatty acid would also be sufficient to produce resistance. Accordingly, at $t=0$ a second addition of acid was made to cells, which had been in contact with acid for the time involved in setting up the manometer vessels, gassing with O_2 and establishing temperature equilibrium, which amounted to about 25–30 min. (Fig. 7). It will be seen that the second addition of acid at $t=0$ decreased the O_2 uptake by about 50% (curve D, Fig. 7), compared with control cells (curves B, C, Fig. 7) and that recovery was delayed for about 30 min., i.e. resistance had not developed during the preliminary incubation period. On the

other hand, 90 min. contact of the cells with a small amount of acid ($R = 0.00625$; $3.7 \times 10^{-6} M$), which by itself did not produce respiratory inhibition (curve *E*, Fig. 7), caused the cells to become resistant. That very small concentrations of substrate, as, for example, $10^{-4} M$ -nitrate or $8 \times 10^{-9} M$ -penicillin, produce marked adaptation in *Escherichia coli* and *B. cereus* respectively, has previously been reported by Wainwright (1950) and by Pollock (1950). Since in the present experiments resistance developed only after a lag period, it seems to depend on some metabolic activity of the cell during that period, which in these experiments, as in those of Pollock, lasted about 1 hr.

Subculture of treated recovered cells. Treated cells have so far only been shown to be resistant to a second treatment with a fatty acid immediately after recovery from the first addition, i.e. without interposition of a period of growth in absence of acid. Experiments were therefore carried out to determine the effect of fatty acids after subculturing recovered cells in absence of fatty acid. In the present arrangement the shortest interval between the removal of the bacteria from the acid-containing medium and re-examination of subcultured cells was 40 hr. The following results were obtained: (1) cells which had developed resistance either as washed suspensions or in a nutrient medium had completely lost their resistance; (2) their O_2 uptake and growth on renewed exposure to any of the fatty acids were inhibited to the same extent and recovery took place at the same rate as that of untreated controls; (3) the process can be repeated many times, apparently indefinitely, without any alteration in the behaviour of the organisms. Whether the complete loss of resistance occurred immediately on subculturing in absence of acid or only gradually could not be decided from the present experiments.

DISCUSSION

The experiments dealing with the inhibition of growth and respiration of *B. subtilis* and the subsequent recovery after treatment with stearic, oleic and linoleic acids differ but little from previous experiments with vaccenic acid (Laser, 1951). The conclusion had then been drawn that the inhibition results from a temporary blocking by penetrated acid of the oxidative system inside the cell and that recovery is due to the ability of the cell to metabolize penetrated acid. It appeared unlikely that physico-chemical surface phenomena could account for the observed facts, especially the recovery from the inhibition and the subsequent resistance of treated cells. The results of the present work make this even more improbable because (1) a second addition of fatty acids still further increases the inhibition, if the acid is added before a certain

degree of recovery by the cells has been attained (curve *D*, Fig. 7); (2) resistance develops also in cells treated with a small dose of acid, which by itself does not cause respiratory inhibition; (3) the addition of fatty acid to treated recovering cells stimulates the O_2 uptake of washed suspensions; (4) treated recovered cells respire and grow at an undiminished rate if transferred into a new medium in presence of fatty acid; (5) the resistance of treated recovered cells is lost after the organisms have been subcultured in absence of added fatty acid. The joint significance of these facts, however, makes it reasonably certain that the phenomenon of resistance is due to the development of an adaptive enzyme. Whether the complete loss of resistance occurred immediately on subculturing in absence of acid or only gradually could not be decided by the present arrangement. However, as resistance had been completely lost, the rate of its loss has no significant bearing on the above conclusion.

Since washed cell suspensions are able to develop resistance, they must be capable of doing so by rearrangement of their protein constituents. That such rearrangements do occur has been clearly demonstrated by Spiegelman & Dunn (1947-8) and Spiegelman & Reiner (1947-8) in their work on the interrelationship of enzymes in bacteria during adaptation, and by Chin (1950), who has described changes of the different components of cytochrome in washed, non-growing yeast cells following upon aeration. The increase in tolerance afforded by a nutrient medium, however, is small, only twofold if, as in these experiments, the substrate concentration is at the tolerance level for washed cells. It is therefore considered likely that the development of resistance in washed cell suspensions and in growing cells, at least in these experiments, differs only in its quantitative aspect, i.e. growth by itself does not confer on the cell a new property. However, where the substrate is either indifferent, as, for example, galactose with *Streptococcus lactis* grown in presence of, and adapted to, glucose, or where it only partly inhibits growth, the process of multiplication can obviously greatly potentiate tolerance and/or enzyme formation. These views are in agreement with those expressed by Spiegelman & Dunn (1947-8) and Spiegelman & Reiner (1947-8), although in the past contradictory conclusions have been drawn by a number of workers (see Yudkin, 1938).

Evidence put forward in this paper, especially the coincidence of the period of inhibition of growth and respiration with the time lag before resistance develops, is believed to support the conclusion that the substrate, towards which the cell ultimately develops resistance, has first to be metabolized by the cell and that the acquired resistance is dependent on this metabolic process.

SUMMARY

1. Stearic, oleic, vaccenic and linoleic acids, within the range of concentrations used, produce an inhibition of respiration of washed suspensions of *Bacillus subtilis* and a concomitant inhibition of respiration and growth of growing cells.
2. The inhibition is transient, its degree and duration being dependent on the ratio acid : cells.
3. Washed suspensions and growing cells which have recovered from the inhibition are resistant to further additions of acid at concentrations higher than the initially inhibiting dose.
4. Treatment with any one acid produces resistance against any of the other acids.
5. A time lag ensues on treatment with a fatty acid before resistance develops.

6. Low concentrations of acid, which do not inhibit growth or oxygen uptake, nevertheless produce resistance.

7. The tolerance of growing cells with subsequent development of resistance is slightly greater than that of washed suspensions.

8. The resistance is lost on subculturing the cells in absence of fatty acid.

9. The evidence suggests that the resistance of acid-treated cells is due to the development of an adaptive enzyme, which is formed subsequently to the incorporation into the cell and the metabolic degradation of the fatty acid.

I am indebted to Dr I. D. Morton for supplying me with the synthetic vaccenic acid.

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The Inhibition of Erythrocyte Cholinesterase by Tri-Esters of Phosphoric Acid

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E600) AND ANALOGUES

BY W. N. ALDRIDGE AND A. N. DAVISON
*Medical Research Council Unit for Research in Toxicology,
 Serum Research Institute, Carshalton, Surrey*

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The ester diethyl *p*-nitrophenyl phosphate (E600) is a powerful and irreversible inhibitor of cholinesterase. The kinetics of its reaction with cholinesterase have been studied (Aldridge, 1950).

The work to be reported here began as a study of the inhibitory powers of analogues of E600 containing different substituents in various positions on the phenyl ring. Comparisons of the activities of other groups of irreversible inhibitors of cholinesterase have been reported. Mackworth & Webb (1948) compared the activity of dialkyl fluorophosphonates prepared by McCombie & Saunders (1946) against a purified preparation of horse-serum cholinesterase. Metcalf & Marsh (1949) compared the activity of substituted diethyl phenyl phosphates and thiophosphates against bee-brain cholinesterase. In these reports a direct comparison was made between

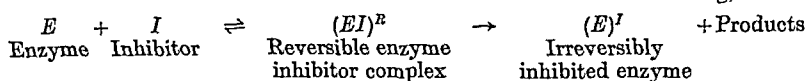
the concentrations of inhibitor that produced 50 % inhibition of the cholinesterase preparation.

Aldridge (1950) showed that the inhibition of erythrocyte cholinesterase by E600 followed first-order kinetics and was bimolecular. The bimolecular rate constants were considered to be a much better index of inhibitory power than the usual 50 % inhibition concentrations. When these constants were determined for this series of compounds it was found that the kinetics of the inhibition were not like those obtained for E600. It was then realized that these differences could be accounted for by the presence of a very active, but unstable, inhibitor of cholinesterase. When this impurity was removed the compounds reacted with cholinesterase in the same way as E600. This aspect of the work seemed to be important, and the work carried out with one of

these compounds is given in detail. With our samples of inhibitors, therefore, the determination of the concentration necessary for 50 % inhibition would have given misleading values for their activity. As will be pointed out, the possibility of the detection of this impurity rested on our choice of cholinesterase preparation and also on a detailed study of the kinetics of inhibition of the enzyme.

A knowledge of the kinetics of inhibition of erythrocyte cholinesterase rests almost entirely on work using E 600. It seemed desirable, therefore, to extend this work to other inhibitors of the same type, for Diggle & Gage (1951) have recently shown that diethyl *p*-nitrophenyl thiophosphate (E 605), purified chromatographically, has an extremely low inhibitory action against cholinesterase. The high inhibitory power of the sample used by Aldridge (1950) must have been due to the impurities it contained. These were most probably the isomers, *OO*-diethyl *S*-*p*-nitrophenyl thiophosphate and *OS*-diethyl *O*-*p*-nitrophenyl thiophosphate.

It was suggested (Aldridge, 1950) that the mechanism of inhibition of cholinesterase could be expressed by the equation:



and that the inhibitor was hydrolysed during the inhibitory process. There is evidence (Jansen, Nutting & Balls, 1949) that when chymotrypsin is inhibited by di-isopropyl fluorophosphonate (DFP), di-isopropyl phosphate remains attached to the inactivated enzyme and that one molecule of acid (presumably HF) is liberated during the inhibitory process. Hartley & Kilby (1950) have shown that one molecule of *p*-nitrophenol is liberated during the inhibition of one molecule of chymotrypsin by E 600. When horse-serum cholinesterase is inhibited by DFP containing ^{32}P phosphorus becomes firmly attached to the cholinesterase (Boursnell & Webb, 1949), and Jandorf & McNamara (1950) have also shown that after a dose of DFP containing ^{32}P to rabbits, the elimination of ^{32}P from the red cells paralleled the reappearance of cholinesterase activity. When mammalian cholinesterase has been prepared in a pure state, a direct experiment to test the hypothesis that the inhibitor is hydrolysed during the inhibitory process may be carried out. At the present time indirect methods must be used, and for this reason it was of interest to compare the stability of hydrolysis at physiological pH and temperature of this series of inhibitors with their inhibitory power. The results confirm the hypothesis stated above.

METHODS

Erythrocytes from defibrinated sheep blood have been used as a source of cholinesterase. Activity has been determined

by the method of Nachmansohn & Rothenberg (1945), as previously described in detail (Aldridge, 1950). With long incubations (2–6 hr.) it was sometimes found that the activity of the control fell appreciably. The addition of 20 $\mu\text{g./ml.}$ of KCN to the buffer for all incubations longer than 1 hr. effectively prevents such loss of activity and does not alter the inhibition by E 600 analogues.

Rates of inhibition have always been determined in double-armed flasks, the red cells in the flask and the inhibitor solution and acetylcholine in the side arms. At zero time, after gassing and temperature equilibration, the inhibitor is tipped into the centre compartment and the incubation finally terminated by tipping in the acetylcholine at the required time.

During the course of this work it was found necessary to have some quantitative measure of reversible and irreversible inhibition. This has been determined by the following washing technique: A red cell suspension of such a concentration of cholinesterase that 0.5 ml. will give an output of approximately 8 $\mu\text{l. CO}_2/\text{min.}$ is incubated with the inhibitor. At the end of the incubation, 5 ml. are pipetted into 45 ml. of buffer in a centrifuge tube and 0.5 ml. directly into 3.0 ml. buffer containing the acetylcholine in a Warburg flask (to determine the total inhibition). The diluted blood is centrifuged at 2500 rev./min. for 5 min., the supernatant removed and the cells washed a further four times with buffer. After the final washing, the cells are resuspended

and diluted to 5 ml. and a sample taken for determination of cholinesterase activity (irreversible inhibition). With cells of low activity larger amounts of blood (up to 3.0 ml.) may be taken, thus increasing the sensitivity six times. Buffer containing 20 $\mu\text{g./ml.}$ of KCN was used for all the washing procedures. Control blood samples with no inhibitor added were always carried through the whole procedure. Stock solutions of all the inhibitors have been prepared in absolute ethanol and dilutions made from them for the experiments.

The following inhibitors, derived from diethyl phosphoric acid, were used: diethyl phenyl phosphate, diethyl *p*- and *o*-chlorophenyl phosphate, diethyl *p*-nitrophenyl phosphate (E 600), diethyl *o*- and *m*-nitrophenyl phosphate and tetraethyl pyrophosphate (TEPP). (*Editorial note.* The names 'diethyl *o*- and *p*-chlorophenyl phosphate' are used in this paper for the sake of consistency with other diethyl phenyl phosphates, although they are contrary to the alphabetical order for prefixes now customary in the *Biochemical Journal*; cf. *J. chem. Soc.* (1950), p. 3699.)

Rates of hydrolysis of the inhibitors in phosphate buffer, pH 7.6, at 37° were determined using the following standard procedure: A weighed amount of ester (approx. 500 mg.) was dissolved in 10 ml. ethanol. At zero time 5 ml. were pipetted into 495 ml. of Sorensen's phosphate buffer (pH 7.6) previously warmed to 37°. As preservatives during the hydrolysis of the *o*- and *p*-nitrophenyl esters, the buffer contained 0.02 % (w/v) thymol while for the rest of the substituted phenyl esters 0.10 % (w/v) merthiolate was used. Samples were withdrawn at various times and the corresponding phenols estimated. *o*- and *p*-Nitrophenol were determined directly by their yellow colour at pH 7.6. Phenol, *o*- and *p*-chlorophenol and *m*-nitrophenol were determined by the method of Gottlieb & Marsh (1946).

The rate of hydrolysis of TEPP was determined manometrically in NaHCO_3 buffer. The volume of CO_2 evolved after complete hydrolysis amounted to 1.94 mol. CO_2 /mol. TEPP.

RESULTS

Washing technique for the determination of irreversible inhibition. As has been shown previously (Aldridge, 1950) intact red cells are a very convenient source of cholinesterase. Inhibitors may be added, allowed to react and then removed by the simple process of washing the cells with buffered saline. The results given in Table 1 show that the cholinesterase activity of normal sheep red cells is not appreciably affected by a prolonged washing equivalent to a dilution of 10^{18} times. If the cholinesterase activity of these cells is inhibited by treatment with E 600, a similar washing process reactivates the enzyme by less than 5%. When red cells are inhibited by eserine, more than 50% of the inhibition is reversed after only one washing (a dilution of 10 times). Our standard procedure for the determination of irreversible inhibition has been to wash five times which is equivalent to diluting 10^5 – 10^6 times.

Table 1. *Washing of sheep red cells before and after treatment with E 600*

(Washed sheep red cells incubated with $6.5 \times 10^{-7} \text{ M}$ E 600 for 30 min. Each washing is equivalent to a dilution of 10 times.)

No. of washings	Activity ($\mu\text{l. CO}_2/\text{min.}$)	
	Without E 600	With E 600
0	9.6	0.7
5	9.4	0.9
18	8.9	1.0

Enzymic hydrolysis of inhibitors. In a preliminary note (Aldridge, 1951) it has been shown that there are enzymes in mammalian tissues which can hydrolyse E 600 and it is, therefore, important to be sure that the cholinesterase preparation used (which is necessarily impure) is free from enzymes which will hydrolyse the inhibitor. This has been checked using E 600, the most unstable to hydrolysis of the substituted diethyl phenyl phosphates we have used. Sheep red cells at a concentration which possessed a cholinesterase activity of 7–10 $\mu\text{l. CO}_2/\text{min.}$ produced a negligible output of CO_2 ($< 0.3 \mu\text{l./min.}$) when E 600 was used as a substrate at a concentration of 2 mg./ml. This means that this preparation is, from a practical point of view, free from any enzyme which will hydrolyse the substituted phenyl inhibitors used in this work. However, red cells catalyse the hydrolysis of TEPP appreciably. When red cells similar to the preparation used above are incubated with TEPP at concentration of about

4 mg./ml., 4 $\mu\text{l. CO}_2/\text{min.}$ are evolved. (This figure has been corrected for the aqueous hydrolysis of TEPP.) As will be explained later inhibition of red-cell cholinesterase by TEPP does not give the kinetics of the other inhibitors and this is due to the destruction of TEPP by the enzyme preparation used. This fact has been of great value in the detection of the impurities in our inhibitors.

Inhibition of cholinesterase by diethyl p-chlorophenyl phosphate. The rate of reaction of the diethyl p-chlorophenyl phosphate has been determined using double-armed flasks so that the inhibitor may be added to the enzyme after temperature equilibration and the reaction stopped later by the addition of acetylcholine from the other side arm. In Fig. 1 are shown the results we obtained. With E 600 first-order kinetics are obtained at each concentration of inhibitor, whereas in this case there is a very rapid, followed by a slow, progressive inhibition.

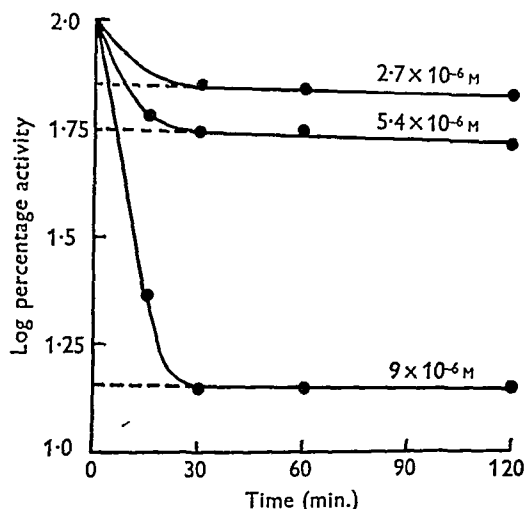


Fig. 1. Rate of inhibition of cholinesterase by unpurified diethyl p-chlorophenyl phosphate. Concentration of inhibitor shown against each curve.

These results were similar to those previously determined with diethyl 8-quinolyl thiophosphate (Aldridge, 1950) when it was shown that the initial rapid inhibition was reversible and could be removed by washing. A determination of irreversible inhibition was, therefore, carried out using the technique described earlier in this paper. As will be seen, in Table 2, none of this initial rapid inhibition is reversible by washing.

Since such closely related compounds as diethyl p-nitrophenyl and p-chlorophenyl phosphates produce such dissimilar results when the rate of their reaction with cholinesterase is examined it seemed probable that impurities were responsible. We should expect the type of kinetics obtained if the rapid irreversible inhibition were produced by a

Table 2. *Washing of red cells after inhibition with diethyl p-chlorophenyl phosphate*

(Cells inhibited for 15 min. at 37°. Each washing is equivalent to a dilution of 10 times.)

Exp. no.		Activity ($\mu\text{l. CO}_2/\text{min.}$)	
		No washes	Five washes
1	Cells (no inhibitor)	8.5	8.7
	Cells + $1.9 \times 10^{-4}\text{M}$ -inhibitor	0.24	0.24
2	Cells (no inhibitor)	7.6	7.8
	Cells + $2.4 \times 10^{-5}\text{M}$ -inhibitor	0.8	0.9

highly active but unstable inhibitor. The highly active inhibitor reacts rapidly with cholinesterase, but is being destroyed so rapidly itself that after about 20–30 min., virtually all of it has been removed. The final slow inhibition would then be due to the diethyl *p*-chlorophenyl phosphate itself. Two observations convinced us that this was probably true. When a chloroform solution of diethyl *p*-chlorophenyl phosphate was shaken with weak caustic soda solution, then with water, dried and the chloroform evaporated, its inhibitory power was considerably reduced. Secondly, a solution of the diethyl *p*-chlorophenyl phosphate which had been incubated at pH 7.6 at 37° for 864 hr., but had only undergone 8% hydrolysis, had a low inhibitory power and when examined for its rate of reaction with cholinesterase gave straightforward kinetics for each concentration tried (Fig. 2).

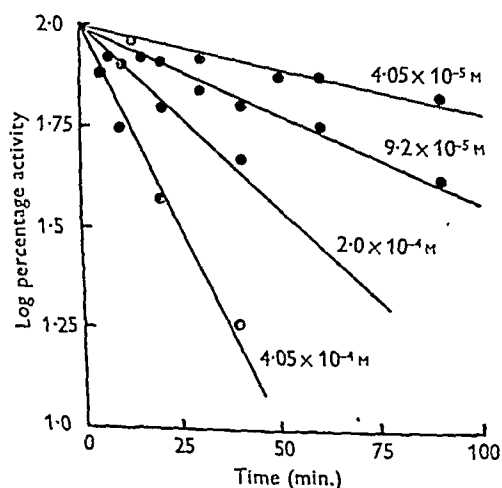


Fig. 2. Rate of inhibition of cholinesterase by purified diethyl *p*-chlorophenyl phosphate. Concentration of inhibitor shown against each curve.

It could be argued that some other change has occurred during its incubation in buffer. In order to check this point, the distribution coefficient of the parent compound between liquid paraffin and water

has been compared with that of the solution of inhibitor after 864 hr. in buffer at pH 7.6 and at 37°. Chloroform, toluene, ether and *n*-amyl alcohol were of little value since the compound was too soluble in them. The results in Table 3 show that the compound in the buffer has the same distribution between liquid paraffin and water as the original compound. If an ethyl group had been removed during the incubation in buffer a di-substituted phosphoric acid would result which would be much more soluble in water. It is clear, therefore, that our sample of inhibitor contains an active inhibitor which is more readily hydrolysed in buffer than is the diethyl *p*-chlorophenyl phosphate.

Table 3. *Distribution coefficient of diethyl p-chlorophenyl phosphate between liquid paraffin and water*

(Concentration of diethyl *p*-chlorophenyl phosphate determined after hydrolysis in NaOH as *p*-chlorophenol using the method of Gottlieb & Marsh (1946). Free *p*-chlorophenol was determined before hydrolysis with NaOH. The solution of inhibitor in phosphate buffer was shaken with an equal volume of liquid paraffin and the concentration of free and bound *p*-chlorophenol repeated on the aqueous layer.)

Compound	Distribution coefficient (room temp.) liquid paraffin/buffer
<i>p</i> -Chlorophenol	1.6
Fresh diethyl <i>p</i> -chlorophenyl phosphate	6.6
Diethyl <i>p</i> -chlorophenyl phosphate after incubation for 864 hr. at pH 7.6 and 37°	6.9

An examination was made of the speed with which this active inhibitor was hydrolysed in water. A solution of the original compound was prepared in the bicarbonate buffer used for the cholinesterase estimations. Samples were withdrawn after 0, 18 and 40 hr. at room temperature and the determination of rate of reaction repeated. In Fig. 3 the results are plotted, and it will be seen that the amount of the rapid initial inhibition decreases rapidly according to the time the inhibitor has been in buffer, until after 40 hr. there is no rapid initial inhibition and the kinetics are first order over the whole experiment. During this period the hydrolysis of the diethyl *p*-chlorophenyl phosphate is negligible.

Our sample of diethyl *p*-chlorophenyl phosphate, therefore, contains an active inhibitor which is sufficiently unstable to be completely hydrolysed after 3 days in bicarbonate buffer at room temperature. However, an examination of Fig. 3 shows that this inhibitor must be removed in 20–30 min. in the presence of sheep red cells. TEPP is an inhibitor which is hydrolysed readily in water, and it has been shown earlier in this paper that the hydrolysis of TEPP is catalysed by sheep red cells and the fact

that the diethyl *p*-chlorophenyl phosphate is a derivative of diethyl phosphoric acid (half of the TEPP molecule) also suggests that TEPP may be the impurity. We have, therefore, added TEPP to a solution of the diethyl *p*-chlorophenyl phosphate which has been incubated in buffer at room temperature for several days and have repeated the determination of rate of reaction with cholinesterase. The results given graphically in Fig. 4 show that these results are similar to those in Fig. 3. Further, if the rate of inhibition of red-cell cholinesterase by TEPP alone is examined, it is found that there is no further inhibition after 30 min. (Fig. 5). It can be argued that this is due to an equilibrium reaction similar to that found with the reversible inhibitor eserine. However, if TEPP is incubated with sheep

that the inactivation is kinetically of the first order and that it is reduced to 2% of its original value, we obtain a first-order constant of 1.6×10^{-3} (min.⁻¹). A measured rate of hydrolysis of TEPP at room temperature under similar conditions gave a value of 1.14×10^{-3} (min.⁻¹). It is, therefore, very

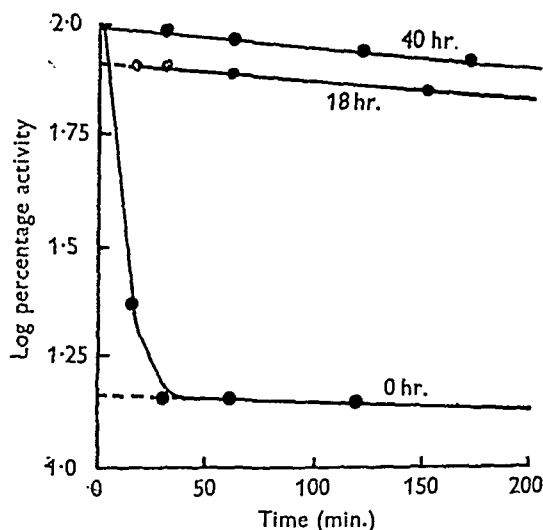


Fig. 3. Rate of inhibition of cholinesterase by 9×10^{-6} M-diethyl *p*-chlorophenyl phosphate after leaving in buffer solution at room temperature. Time in buffer shown against each curve.

red cells for 30 min., the cells centrifuged and the supernatant added to fresh red cells, the cholinesterase activity of these red cells is not inhibited; therefore, there is no TEPP remaining in solution and this is presumably due to its enzymic hydrolysis.

It is proved, therefore, that our sample of diethyl *p*-chlorophenyl phosphate contains as an impurity an active cholinesterase inhibitor which is completely inactivated within 3 days at room temperature in bicarbonate buffer and which is inactivated by sheep red cells at low concentrations in 20–30 min.; this substance is in all probability TEPP, since the addition of TEPP to diethyl *p*-chlorophenyl phosphate from which the active inhibitor has been removed by preferential hydrolysis, gives, on examination, the same kinetic picture as the original diethyl *p*-chlorophenyl phosphate. It has been shown that the inhibitor is reduced to a very low level by 40 hr. If it is assumed

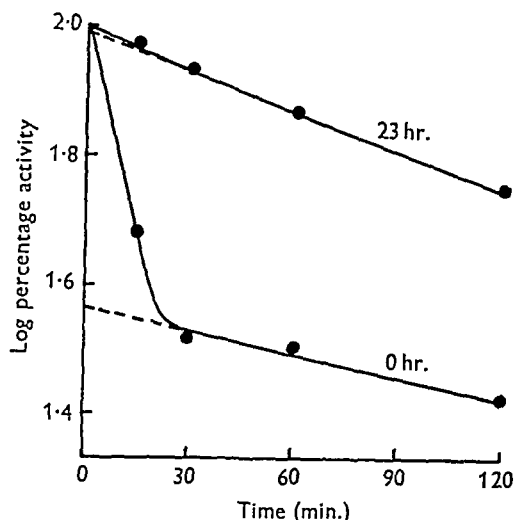


Fig. 4. Rate of inhibition of cholinesterase by 2.7×10^{-8} M-TEPP plus 3.97×10^{-5} M-purified diethyl *p*-chlorophenyl phosphate, at zero time and after 23 hr. in bicarbonate buffer at room temperature.

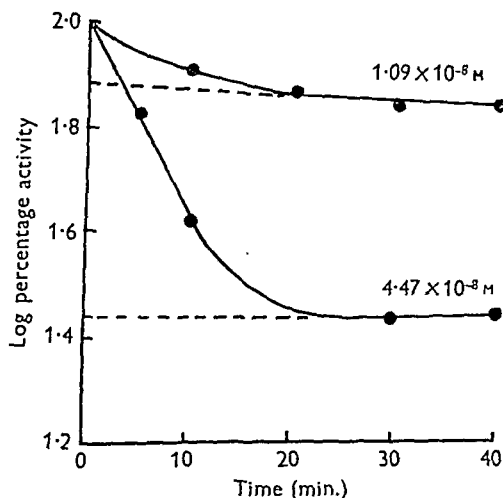


Fig. 5. Rate of inhibition of cholinesterase by TEPP. Concentration of inhibitor shown against each curve.

probable that the impurity present is TEPP and the amount of impurity will be expressed as TEPP (cf. Table 6).

The determination of concentration of TEPP in inhibitors. Aldridge (1950) has shown that the inhibition of red-cell cholinesterase by E 600 shows first-order kinetics and is bimolecular, i.e.

$$K = \frac{1}{tI} \ln \frac{100}{b}, \quad (1)$$

where K = bimolecular rate constant, t = time in min., I = molar inhibitor concentration and b = percentage residual activity. If the time is maintained constant at 30 min. a plot of $\log b$ against I , the inhibitor concentration, should give a straight line. This graphical method can be conveniently used for the determination of the concentration of an inhibitor from its inhibitory power against cholinesterase. TEPP has been shown to be destroyed during the incubation with red cells. If it is assumed that the kinetics of the inhibition of cholinesterase by TEPP are similar to those obtained for E 600 and that the kinetics of the hydrolysis of TEPP during the incubation period are also first order (cf. Van Slyke, 1942, for a discussion of enzyme kinetics at low substrate concentrations), then a plot of $\log \%$ residual activity against inhibitor concentration should give a straight line. This is found to be so (Fig. 6).

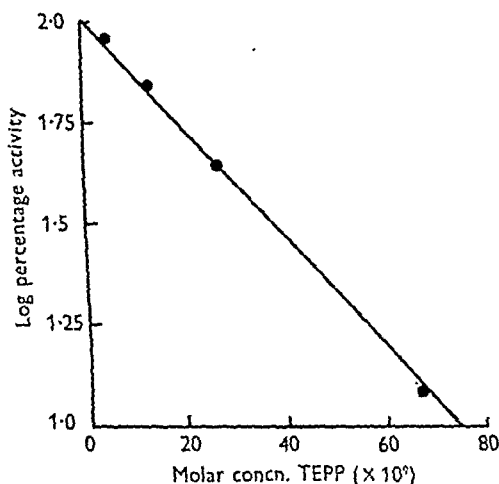


Fig. 6. Inhibition of sheep red cells by TEPP incubated for 30 min. at 37°.

The amount of inhibition due to TEPP in our diethyl *p*-chlorophenyl phosphate may be readily determined by extrapolating the straight line portion of the curves (after 30 min.) back to the ordinate. The amount below the origin will give a measure of the inhibition due to TEPP. This has been checked using the results obtained with our synthetic mixture of TEPP and purified diethyl *p*-chlorophenyl phosphate (Fig. 4). At zero time an extrapolation in this way shows that the line crosses the ordinate at 1.59 which from Fig. 6 is equivalent to 3.0×10^{-8} M-TEPP; the amount added was 2.7×10^{-8} M. This method has been used to obtain an estimate of the amount of TEPP present in our inhibitors. Our main purpose has been to show that the amounts present are too small to be detected by analysis of the original inhibitor and could only be determined by a biochemical method.

Calculation of bimolecular rate constants. If an inhibitor reacts bimolecularly with an enzyme, equation (1) shows that with the concentration of inhibitor (I) constant, a straight line will be obtained when $\log \%$ residual activity is plotted against time, i.e. the system will show first-order kinetics. If the inhibitor concentration (I) is varied then $\log \%$ residual activity as ordinates plotted against the product of time and concentration (tI) as abscissae will give a straight line crossing the ordinate at 2.0 (100% activity). For all of the compounds (except TEPP) examined the point where the line cuts the ordinate has been determined and shown to be 2.0 within experimental error. This is illustrated by Figs. 2 and 7 using our results on diethyl *p*-chlorophenyl phosphate.

Examination of other inhibitors. Each inhibitor has been examined by the technique previously described for diethyl *p*-chlorophenyl phosphate

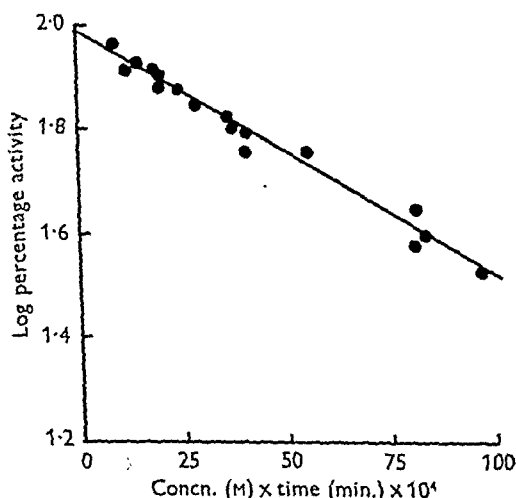


Fig. 7. Graph of all results for cholinesterase inhibition by purified diethyl *p*-chlorophenyl phosphate. Bimolecular rate constant derived from slope of line is 1.1×10^2 ($\text{min.}^{-1} \text{ l. mol.}^{-1}$).

(Fig. 3). Except for E 600 the presence of a similar active and unstable inhibitor has been shown. It has been assumed that this is TEPP. The concentrations for 50% inhibition (after 30 min. incubation at 37°) for the original compound, and after it has been purified (by preferential hydrolysis), are calculated as previously described and are given in Table 4.

The amount of impurity (expressed as TEPP) present in all (except phenyl (2)) of these samples of inhibitor is below 0.7%; this concentration would be undetectable by the methods of analysis.

The inhibition of cholinesterase by all of the purified inhibitors given in Table 4 has been shown to give first-order kinetics and to be bimolecular by the methods previously illustrated using diethyl

p-chlorophenyl phosphate. Using these data, the bimolecular rate constants have been calculated and are given in Table 5. The value given for TEPP is one corrected for hydrolysis of TEPP during the experiment on the assumption that this hydrolysis will be first order and that the TEPP concentration is reduced to 95% of its original value in 30 min. Also included in Table 5 are the first-order constants for aqueous hydrolysis at pH 7.6 and 37° in phosphate buffer.

Table 4. Concentrations for 50% inhibition by unpurified and purified inhibitors and the concentration of TEPP present as impurity

(Samples purified by preferential hydrolysis of impurity for 3-4 days at room temperature in bicarbonate buffer. Concentrations of TEPP calculated as given in the text.)

Inhibitor	Concn. for 50% inhibition (M)		Impurity (TEPP) (%)
	Unpurified	Purified	
<i>p</i> -Chloro-	5.75×10^{-6}	2.10×10^{-4}	0.68
<i>o</i> -Chloro-	2.24×10^{-5}	1.1×10^{-4}	0.053
<i>p</i> -Nitro-(E 600)	2.0×10^{-8}	2.01×10^{-8}	—
<i>o</i> -Nitro-	4.58×10^{-7}	1.48×10^{-6}	0.33
<i>m</i> -Nitro-	1.2×10^{-6}	3.0×10^{-6}	0.55
Phenyl (1)	1.35×10^{-4}	3.77×10^{-3}	0.025
Phenyl (2)	2.35×10^{-7}	3.77×10^{-3}	5.4

The results in Table 5 indicate that the more stable the inhibitor the slower is its inhibitory action upon cholinesterase. These results have been plotted in Fig. 8 where the relation between stability of an inhibitor in buffer at physiological pH and temperature and its inhibitory power is shown.

TEPP and E 600 appear to be exceptional. This might be expected with TEPP, since it has a different chemical structure to the rest of the inhibitors. However, E 600 appears to have a far higher inhibitory power than would be expected from its rate of hydrolysis. E 600 and diethyl *o*-nitrophenyl phosphate have roughly the same stability at pH 7.6 and 37°, but E 600 is 70 times more efficient as an inhibitor. There is no point in speculating

why this might be so until more inhibitors of this type with rate constants higher than 10^4 (l.mol.⁻¹ min.⁻¹) have been examined. It is of interest that Metcalf & Marsh (1949), in work on bee-brain cholinesterase, found E 600 to be an outstanding inhibitor in a large series of compounds.

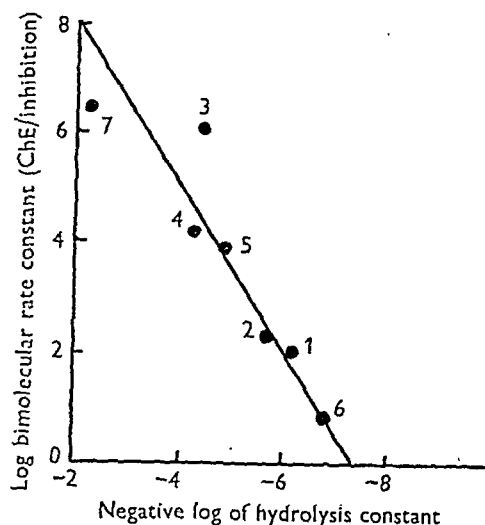


Fig. 8. Relationship between rate of hydrolysis in buffer at pH 7.6 and 37° and the bimolecular rate constant for the reaction of cholinesterase (ChE) with inhibitor. Compounds numbered as in Table 5.

DISCUSSION

The work described above began with an attempt to compare the rate of hydrolysis at pH 7.6 and 37° and the rate of reaction with cholinesterase of analogues of E 600. During the initial experiments it was found that the reaction of all these analogues with cholinesterase had peculiar kinetics quite unlike E 600. A further examination led to the idea that an unstable active inhibitor was present as an impurity. This view had been substantiated. The impurity appeared to be the same in all of our compounds (based on rate of destruction in bicarbonate buffer) and therefore it is unlikely that it could have

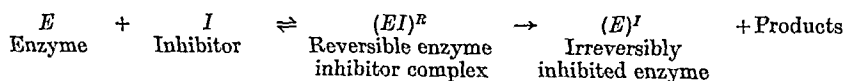
Table 5. Constants for rate of reaction of inhibitors with cholinesterase and for their hydrolysis in buffer

(Since most of the compounds are very stable, the long time required for their complete hydrolysis made necessary the calculation of the non-enzymic hydrolysis constants from data involving incomplete hydrolyses. The proportion of the compound hydrolysed for the period during which the determinations were made is given in brackets. ChE = cholinesterase. Numbers of compounds refer to points in Fig. 8.)

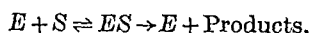
Inhibitor	No.	Rate of reaction of inhibitor with ChE K (min. ⁻¹ l.mol. ⁻¹)	Rate of hydrolysis of inhibitor in phosphate buffer, pH 7.6, at 37° K (min. ⁻¹)
<i>p</i> -Chloro-	1	1.1×10^2	7.2×10^{-7} (4.2%)
<i>o</i> -Chloro-	2	2.1×10^2	2.3×10^{-6} (7.9%)
<i>p</i> -Nitro-(E 600)	3	1.1×10^5	3.9×10^{-5} (65%)
<i>o</i> -Nitro-	4	1.6×10^4	5.6×10^{-5} (78%)
<i>m</i> -Nitro-	5	7.7×10^3	1.4×10^{-5} (45%)
Phenyl	6	6.1	1.6×10^{-7} (2.9%)
TEPP	7	3.3×10^5	6.0×10^{-3} (100%)

been an impurity common to all the substituted phenols used in the synthesis of the compounds. All the analogues are derivatives of diethyl phosphoric acid and this suggested that tetraethyl pyrophosphate might be the impurity. The following evidence confirms this view: (1) reasonable agreement between the rate of hydrolysis of TEPP and the rate of disappearance of impurity in bicarbonate buffer; (2) sheep red cells remove TEPP in the same time (25–30 min.) as that taken to remove the impurity; and (3) a model system in which TEPP was added to the purified diethyl *p*-chlorophenyl phosphate produced the same kinetic picture as that of the unpurified inhibitor.

After purification by leaving a solution of the compound in bicarbonate buffer at room temperature for 2–3 days, the kinetics of the rate of reaction of these inhibitors with cholinesterase was re-examined. They all now followed first-order kinetics at any given inhibitor concentration and by the usual tests the reactions were bimolecular. The bimolecular rate constants for these reactions have been determined and have been shown in general to run parallel to the rate of hydrolysis of the inhibitors in water. The more unstable the compound, the more active it is as an inhibitor. This is evidence in favour of the view that hydrolysis is a part of the inhibitory process. The mechanism originally suggested (Aldridge, 1950),



is the same as that of the Michaelis-Menten equation for the enzyme-substrate system,



The two processes of inhibition and hydrolysis of substrate are probably analogous. The enzyme may form a complex with the inhibitor, activate it and the inhibitor is then hydrolysed. In the case of the inhibitor the active centre is blocked by the retention of one of the products of hydrolysis. The final proof of this hypothesis could only come with a sufficiently pure and concentrated preparation of cholinesterase, so that the products of this reaction could be produced in quantities great enough to be determined chemically.

Intact red cells have been used and therefore the cholinesterase is not in solution and the system is obviously a heterogeneous one. This reaction of inhibitor and enzyme differs from most other surface reactions in the important respect that the active surface is itself being inactivated. It is probable that the concentration of even the most powerful inhibitor (10^{-8} M E 600) is considerably in excess of the concentration of enzyme considered in terms of

its active centres. On this assumption, therefore, the concentration of inhibitor will be sensibly constant throughout the reaction, and first-order kinetics can be expected. The frequency of a fruitful collision of inhibitor and active centre (i.e. a collision which leads to irreversible inhibition of the active centre) will be dependent upon the number of available active centres, and since these will be continuously depleted by the reaction itself, an exponential reaction rate will result.

Finally, a consideration of the conditions which enabled us to detect the impurity in our compounds will illustrate some of the possible sources of error to be considered when a value for the inhibitory power of these compounds is determined. The detection of the impurity depended on its instability and hydrolysis by enzymes present in our preparation of cholinesterase. In fact, if we had been able to use a pure preparation of cholinesterase we should have obtained the expected kinetics. These would have been attributed to the particular inhibitor being examined, though in fact they were really those of the active impurity. Since they have activities of the same order TEPP cannot be detected in E 600. Only high concentrations of TEPP would have altered the kinetics obtained with E 600 and this could have been detected by the normal methods of chemical analysis. A stable inhibitor present as an impurity could not be detected. For instance we

could not trace by these methods E 600 in the diethyl *o*- and *p*-nitrophenyl phosphates. This possibility can be minimized by using substituted phenols of high purity in the preparation of the inhibitors. These results suggest the need for care in comparing the activity of inhibitors when concentrations of impurities undetectable by ordinary chemical means may produce large changes in a biochemical system. The value of a complete study of the kinetics of the reaction between enzymes and inhibitors is illustrated.

SUMMARY

1. Kinetics of inhibition of sheep red-cell cholinesterase by six substituted diethyl phenyl phosphate inhibitors have been examined and shown to be first order and bimolecular.

2. All the inhibitors examined contained as an impurity a small amount of an active and unstable inhibitor of cholinesterase. This is in all probability tetraethyl pyrophosphate. A simple method of purification is given and the methods used to detect and determine tetraethyl pyrophosphate are given in detail.

3. A relation between the stability of the inhibitors to aqueous hydrolysis at physiological pH and temperature and their power as inhibitors of cholinesterase has been found. The more stable the inhibitor, the lower its inhibitory power.

4. The mechanism of inhibition of cholinesterase

by these compounds is discussed in relation to the above facts.

Our thanks are due to Mr B. Topley, Messrs Albright and Wilson Ltd., for providing us with the samples of all the inhibitors used in this work, and to Miss J. I. Wheatley for valuable technical assistance.

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The Metabolism of Aminophenols, *o*-Formamidophenol, Benzoxazole, 2-Methyl- and 2-Phenyl-benzoxazoles and Benzoxazolone in the Rabbit

By H. G. BRAY, R. C. CLOWES AND W. V. THORPE

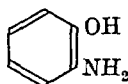
Department of Physiology, Medical School, University of Birmingham

(Received 28 August 1951)

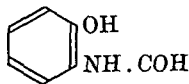
In this investigation some aspects of the metabolism of *o*-, *m*- and *p*-aminophenols, *o*-formamidophenol (II), benzoxazole (III), 2-methylbenzoxazole (IV), 2-phenylbenzoxazole (V), and benzoxazolone (VI) have been examined. The compounds (II) to (VI)

benzoxazolone, which Gressly & Nencki (1890, 1891) showed to be hydroxylated in the dog.

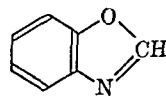
In this study we have attempted to draw up balance sheets for these compounds and to isolate their principal metabolites. Minor metabolites



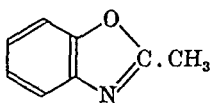
(I)



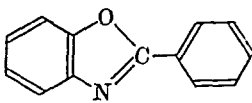
(II)



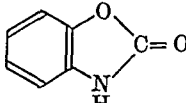
(III)



(IV)



(V)



(VI)

can be regarded as derivatives of *o*-aminophenol (I), which might be formed from them on hydrolysis. These compounds may, therefore, be considered as having potential centres for conjugation (see Bray, Ryman & Thorpe, 1948; Thorpe, 1950). Williams (1938, 1943) and Smith & Williams (1949) found that the rabbit excretes the aminophenols mainly as *O*-conjugates, i.e. ethereal sulphates and glucuronides. The only other compound of this group which appears to have been investigated previously is

were detected by means of paper chromatography. The results obtained show the influence of substituents upon the stability of the oxazole ring.

EXPERIMENTAL

Materials. The aminophenols were purchased (British Drug Houses Ltd. and Light and Co.). *o*-Formamidophenol was prepared by the action of acetic and formic acids on *o*-aminophenol (Béhal, 1900), benzoxazole and its 2-methyl and 2-phenyl derivatives by dry distillation of *o*-amino-

phenol with formamide, acetamide and benzamide respectively (cf. Bamberger, 1903), benzoxazolone by the method of McDonald & Chechak (1948), *o*-hydroxyphenylurea by the method of Menschutkin (1870) and *o*-aminophenylsulphate by the method of Burkhardt & Wood (1929). 2:4-Dihydroxybenzamide (m.p. 227–228°) was prepared from 2:4-dihydroxybenzoic acid (British Drug Houses Ltd.) by heating the methyl ester with NH_3 in a sealed tube.

Diet and dosage. The rabbits used (does of 2–3 kg. weight) were maintained throughout the investigation on the diet of rabbit pellets and water customary in this laboratory (Bray, Ryman & Thorpe, 1947). The compounds studied were administered by stomach tube as suspensions or emulsions in water in doses of 1 g. Some rabbits exhibited a mild paralysis of short duration after receiving *o*- and *p*-aminophenol and benzoxazolone. Administration of benzoxazolone was often followed by the excretion of amounts of reducing material far in excess of that which could be formed as metabolites of the compounds given. This was also observed, but less frequently, after giving *o*-aminophenol. Similar excretion of reducing material has been observed with other compounds (see Bray, Hybs & Thorpe, 1951). No toxic symptoms were observed with the other compounds administered.

Methods. None of the compounds studied had any specific property suitable for quantitative estimation of the individual compound. Ethereal sulphate was determined by the method of Folin (1905–6), glucuronic acid by a modification of that of Hanson, Mills & Williams (1944), ether glucuronide by the reducing value method (Bray, Neale & Thorpe, 1946; Bray, Lake, Neale, Thorpe & Wood, 1948), ether-soluble acid as described by Bray, Thorpe & Wood (1951) but using bromothymol blue as indicator, diazotizable compounds by the method of Bratton & Marshall (1939), amino compounds by the Ehrlich reaction (Venkataraman, Venkataraman & Lewis, 1948; Bray, Thorpe & Wood, 1951), phenols by the

Folin & Ciocalteu reaction (Bray, Thorpe & White, 1950*b*), and mercapturic acids by a modification of the method of Stekol (1936). The Hilger Spekker photo-absorptiometer was used for colorimetric estimations.

Urine extracts were examined by paper chromatography as described by Bray, Thorpe & White (1950*a*). The solvent mixtures and the R_F values of the reference compounds used are given in Table 1.

Calculation of results. The chief compounds which might be expected to be present in urine after administration of *o*-, *m*- or *p*-aminophenol are: the unchanged aminophenol, *A*; the acetamidophenol, *B*; the aminophenylsulphate, *C*; the acetamidophenylsulphate, *D*; the aminophenylglucuronide, *E*, and the acetamidophenylglucuronide, *F*. The free aminophenols give no appreciable colour when diazotized and coupled under the Bratton & Marshall (1939) conditions, but they can be estimated by the modified Ehrlich method. *o*-Aminophenylsulphate, while stable in N-HCl in the cold, is decomposed giving sulphate ions on addition of the nitrite for diazotization and gives no colour with the Bratton & Marshall reagent. *p*-Aminophenyl sulphate is more stable and can be diazotized and coupled without losing sulphate (Burkhardt & Wood, 1929). Heating the urine in a boiling-water bath with HCl (0.2 vol. 2*N*) for 30 min. (mild hydrolysis) was found to effect the hydrolysis of the acetamido and sulphate groups, but was not sufficient to liberate glucuronic acid (i.e. these were the conditions which gave maximum diazo values). Hydrolysis of glucuronide was only achieved by heating in a boiling-water bath with HCl (0.33 vol. 10*N*) for 90 min. (complete hydrolysis of conjugates). By carrying out determinations upon the urine as collected, and after mild or complete hydrolysis the following data were obtained from which the amounts of the compounds *A–F* present could be calculated. Diazo determination on urine as collected, *E* or *C + E* (according

Table 1. Paper chromatography of various phenolic compounds

(Whatman no. 4 paper. Solvent mixture: *A*, *n*-butanol, 40; pyridine, 20; saturated aqueous NaCl , 25; ammonia, sp.gr. 0.880, 15 (all % v/v); *B*, benzene saturated with formic acid (98–99%); *C*, benzene, 40; glacial acetic acid, 40; water, 20; *D*, chloroform, 40; glacial acetic acid, 40; water, 20. Time 4 hr., except for mixture *B*, 16 hr.)

Compound	R_F value in solvent mixture				Colour given by diazotized <i>p</i> -nitraniline*
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	
<i>o</i> -Aminophenol†	0.8	0	0.05	0.45	
<i>m</i> -Aminophenol†	0.75	0	0.04	0.40	Olive green
<i>p</i> -Aminophenol†	0.75	0	0.02	0.40	Red-brown
<i>o</i> -Formamidophenol	0.7	0.5	0.7	1.0	Dark brown
<i>o</i> -Acetamidophenol	0.75	0.75	0.8	1.0	Violet
<i>o</i> -Benzamidophenol	0.9	0.95	0.9	1.0	Violet
<i>m</i> -Acetamidophenol	0.8	0.1	0.30	0.9	Violet
<i>p</i> -Acetamidophenol	0.9	0.85	0.25	0.9	Purple
<i>m</i> -Hydroxybenzoic acid	0.1	0.2	0.7	0.9	Blue
<i>p</i> -Hydroxybenzoic acid	0.05	0.2	—	0.9	Crimson
<i>p</i> -Hydroxyphenylacetic acid	0.1	0.15	0.6	0.9	Crimson
<i>o</i> -Hydroxyphenylurea	0.65	0.15	0.4	0.95	Violet
Hydroxybenzoxazolone‡	0.6	0.1	0.2	0.75	Mauve
2-(3-Hydroxyphenyl)-benzoxazole‡	0.9	0.95	—	—	Blue-green
Unidentified compound from 2-methylbenzoxazole urine	0.65	0.15	0.3	—	Purple
Chloro-2:4-dihydroxybenzamide	—	—	0.18	—	Purple

* See Bray *et al.* (1950*a*).

† These compounds reduce ammoniacal AgNO_3 .

‡ These compounds were isolated from urine.

to stability of the aminophenylsulphate). Diazo determination on urine after mild hydrolysis, $E + F$. Ehrlich determination on urine as collected, $A + C + E$. Ehrlich determination on urine after complete hydrolysis,

$$A + B + C + D + E + F.$$

Ethereal sulphate determination, $C + D$. Glucuronic acid determination (naphthoresorcinol), $E + F$. It can be seen from Table 2 that glucuronide results are in reasonable agreement with those found by diazo determination on the urine after mild hydrolysis. The relative proportions of E and F were determined by diazo estimations, before and after hydrolysis, on the glucuronide fraction of the pooled urines of ten rabbits obtained by the usual lead procedure (cf. Bray *et al.* 1947). Any significant difference (such differences were only obtained when benzoxazoles were administered) between the $E + F$ value estimated by diazotization and by the naphthoresorcinol method would suggest the existence of a glucuronide in which there is no diazotizable amino group, e.g. that of a hydroxylated benzoxazole. Even if the oxazole ring of such a compound was ruptured during hydrolysis the resulting dihydroxy-aniline would not be expected to diazotize and couple, since the liberated amino group would be in a position *ortho* to hydroxyl.

RESULTS

Excretion of normal metabolites. The average daily excretion of various metabolites by individual rabbits ranged as follows: ethereal sulphate, 26–41 mg. SO_3 ; reducing material, unhydrolysed, 175–210 mg., hydrolysed, 305–480 mg. (calculated as glucuronic acid); ether-soluble acid, 604–900 mg. (calculated as hippuric acid); phenols, free, 25–

60 mg. (using 2-(3-hydroxyphenyl)-benzoxazole as standard) or 17–41 mg. (using hydroxybenzoxazolone as standard), conjugated, 39–97 mg. (calculated as hydroxybenzoxazolone); mercapturic acid, 4–22 mg. (calculated as mercapturic acid of benzoxazolone). The average percentages by which individual daily normal values differed from the weekly average values used for baselines in calculation of final results were ± 14 , ± 10 , ± 10 , ± 11 , ± 29 or 37 , ± 18 and ± 65 respectively.

Metabolism of *o*-, *m*- and *p*-aminophenols

Quantitative. The results of determinations on urines excreted by rabbits which had received *o*-, *m*-, or *p*-aminophenol are summarized in Table 2. From these values the amounts of the different metabolites excreted have been calculated and are given in Table 3. The following illustrates the method of calculation for *o*-aminophenol. From diazo estimation on urine as collected $E = 53\%$. Hence, since $E + F = 65\%$, $F = 12\%$. Calculation from the ratio free : acetylated glucuronide (3.8:1) gives 51 and 14%. Values taken for E and F were, therefore, 52 and 13% respectively. $C + D = 15\%$ and from chromatographic estimation (Table 3) $A = 11\%$. Hence, since

$$A + B + C + D + E + F = 95\%, \quad B = 4\%.$$

Since *o*-aminophenylglucuronide gives only 65% and *o*-aminophenylsulphate only 33% of the colour

Table 2. Results of quantitative analysis of urines from rabbits dosed with *o*-, *m*- and *p*-aminophenols

(Results are expressed as average percentage of the dose; ranges in parentheses. Superior figures against ranges indicate the number of experiments. Dose 1 g. throughout.)

Method of estimation	<i>o</i> -Aminophenol	<i>m</i> -Aminophenol	<i>p</i> -Aminophenol
Diazo* on urine as collected (E or $C + E$)	53 (37–60) ¹⁰	59 (54–63) ⁶	53 (45–65) ⁸
Diazo* on urine after mild hydrolysis ($E + F$)	65 (60–68) ⁵	64 (62–67) ⁶	61 (55–65) ⁶
Ehrlich† on urine as collected ($A + C + E$)	50 (41–70) ¹⁰	58 (56–61) ⁶	79 (61–96) ⁸
Ehrlich† on urine after complete hydrolysis ($A + B + C + D + E + F$)	95 (86–108) ¹⁰	98 (85–118) ⁶	104 (85–125) ⁸
Ethereal sulphate ($C + D$)	15 (13–17) ²	15 (14–15) ²	12 (10–13) ²
Glucuronic acid ($E + F$)	70 (62–77) ^{2†}	60 (58–61) ²	62 (60–63) ²
Ratio of free to acetylated glucuronide in glucuronide fraction of urine ($E:F$)	3.8:1	10.8:1	2.8:1

* Corresponding aminophenylglucuronide used as standard.

† The aminophenol used as standard. The *o*-, *m*- and *p*-aminophenylglucuronides gave respectively 65, 105 and 180% of the colour intensity of the corresponding aminophenol.

‡ Pure *o*-aminophenylglucuronide gave only 80% of the theoretical colour value with the naphthoresorcinol reagent. These values have, therefore, been corrected. In these two experiments the dose did not cause the excretion of large amounts of reducing material (see p. 75).

Table 3. Amounts of metabolites excreted by rabbits after administration of aminophenols

(Results are expressed as average percentages of the dose and are calculated from data of Table 2 except for values in parentheses which were obtained by paper chromatography. Dose 1 g. throughout.)

Metabolite	<i>o</i> -Aminophenol	<i>m</i> -Aminophenol	<i>p</i> -Aminophenol
Unchanged aminophenol	(11)	0 (0)	(2)
Acetamidophenol	4 (2)	19 (12)	25 (13)
Aminophenylsulphate	15	0	8
Acetamidophenylsulphate	0	15	4
Aminophenylglucuronide	52	59	45
Acetamidophenylglucuronide	13	5	16

intensity of *o*-aminophenol in the Ehrlich method, the determination on urine as collected really gives $A + 0.33C + 0.65E$. Hence

$$11 + 0.33C + 0.65 \times 52 = 50$$

and $C = 15\%$ and $D = 0$. For *p*-aminophenol, C can be obtained from $C + E = 53$, assuming that the intensity of colour given by *p*-aminophenylsulphate in the diazo method is the same as that given by *p*-aminophenylglucuronide. The intensity of colour in the Ehrlich method for *m*-aminophenylglucuronide is the same as that for *m*-aminophenol, hence both A and $C = 0$.

The presence of aminophenol and acetamidophenol in ether extracts of the urines was detected by two-dimensional paper chromatograms using solvent mixture *A* followed by solvent mixture *B* (Table 1). Approximate estimations of the amounts present were made from one-dimensional chromatograms using solvent mixtures *A* (*o*- and *p*-aminophenol), *B* (*m*-acetamidophenol) and *C* (*o*- and *p*-acetamidophenol). The results are given in parentheses in Table 3.

Isolation of metabolites. The urines as collected (pH 7–8) were extracted with ether for 24 hr. in a continuous extractor. The following were isolated and characterized: *o*-aminophenol, m.p. 173° (7.5% of dose), from *o*-aminophenol urine; *m*-acetamidophenol, m.p. 148° (6% of dose); from *m*-aminophenol urine and *p*-acetamidophenol, m.p. 168° (4% of dose), from *p*-aminophenol urine. The amounts isolated provide confirmation of the relative amounts of free and acetylated phenols found by quantitative methods (Table 3).

Crystalline glucuronides were isolated from the urines by means of the usual lead procedure (cf. Bray *et al.* 1947). Their properties, given in Table 4, were essentially in agreement with those recorded by Williams (1943). The *o*-aminophenylglucuronide which we isolated appeared to be anhydrous. (Found: C, 50.1; H, 5.7; N, 5.0, 4.6; equiv. 265. Calc. for $C_{12}H_{15}O_7N$: C, 50.5; H, 5.3; N, 4.9%; equiv. 285.)

Table 4. *Properties of the glucuronides isolated from urine of rabbits after administration of aminophenols*

	<i>o</i> -Aminophenyl- glucuronide. Colourless elongated platelets	<i>m</i> -Aminophenyl- glucuronide. Colourless leaflets	<i>p</i> -Aminophenyl- glucuronide. Colourless needles
Crystalline form			
Melting point	Blackens from 230°*	Blackens from 220°	218° (decomp.)†
$[\alpha]_D$ in water (<i>c</i> , 0.5)	–87° at 20°	–100° at 21.5°	–93° at 21°
N% (calc. 4.9)	4.8	4.6	5.1
Equiv. by titration (calc. 285)	265	290	293
Yield (% of dose)	24	6	18

* Williams (1943) gives 'blackened without melting on heating to 300°'. Comparison of our glucuronide with a specimen kindly provided by Prof. Williams showed identical behaviour of the two specimens in the melting-point apparatus, namely, no true melting but blackening starting about 230°. Prof. Williams states that by 'blackened without melting on heating to 300°' he meant that the 'limit of heating was 300°', and not that it blackened specifically at 300°.

† Williams (1943) gives 213° (decomp.) for monohydrate. (Calc. for monohydrate: N, 4.6%; equiv. 303.)

Metabolism of o-formamidophenol

Metabolites which might be expected to be excreted after administration of *o*-formamidophenol are *o*-formamidophenylsulphate and *o*-formamidophenylglucuronide, together with the unchanged compound and the metabolites of *o*-aminophenol described in the previous section. The quantitative methods used for aminophenol urines were applied and the results are summarized in Table 5. *o*-Formamidophenol neither diazotizes and couples nor reacts with the Ehrlich reagent, but it forms *o*-aminophenol on mild hydrolysis. The methods do not permit differentiation between the formamido and acetamido compounds. With this limitation the amounts of the various metabolites excreted have been calculated and are given in Table 6.

o-Aminophenol, *o*-formamidophenol and *o*-acetamidophenol were detected in ether extracts of the urine by two-dimensional paper chromatography using solvent mixtures *A* and *B* (Table 1). Approximate estimations indicated an amount of *o*-aminophenol corresponding to 2% of the dose and of *o*-acetamidophenol to 1%. Owing to the difficulty of finding a solvent mixture which separated it clearly from the phenols present in normal urine the amount of *o*-formamidophenol could only be assessed as between 5 and 10% of the dose.

Isolation of metabolites. The urine as collected (pH 7–8) from rabbits which had been given *o*-formamidophenol was continuously extracted with ether. The only metabolite isolated from the extract was *o*-formamidophenol. Yield 2.5% of the dose. *o*-Aminophenylglucuronide was isolated from the urine by the usual lead procedure. Yield 8% of dose.

Metabolism of benzoxazole

If the metabolism of benzoxazole in the rabbit involves rupture of the oxazole ring, *o*-formamidophenol and *o*-aminophenol are likely fission products. The urines from rabbits which had received benzoxazole were, therefore, treated like the

Table 5. *Results of quantitative analysis of urines from rabbits dosed with o-formamidophenol, benzoxazole and 2-methylbenzoxazole*

(Results are expressed as average percentage of dose; ranges given in parentheses. Superior figures indicate number of experiments. Dose 1 g. throughout.)

Method of estimation	<i>o</i> -Formamidophenol	Benzoxazole	2-Methylbenzoxazole
Diazo* on urine as collected	54 (39-62) ⁶	37 (31-47) ¹⁰	7 (5-10) ⁸
Diazo* on urine after mild hydrolysis	74 (68-77) ⁵	50 (42-56) ⁸	35 (26-42) ⁸
Ehrlich† on urine as collected	39 (35-44) ⁶	25 (14-39) ¹⁰	7 (3-8) ⁸
Ehrlich† on urine after complete hydrolysis	107 (90-131) ⁶	98 (87-132) ⁶	99 (83-112) ⁸
Ethereal sulphate	16 ²	16 (14-19) ⁴	16 (15-17) ²
Glucuronic acid	60 (57-63) ²	64 (52-83) ⁶	67 (55-76) ⁶
Ratio of free to acetylated glucuronide	2:1	2.3:1	1.4:2.5

* *o*-Aminophenylglucuronide used as standard.

† *o*-Aminophenol used as standard.

Table 6. *Amounts of metabolites excreted by rabbits after administration of o-formamidophenol, benzoxazole and 2-methylbenzoxazole*

(Results are expressed as average percentages of the dose and are calculated from data of Table 5, except for the values in parentheses, obtained by paper chromatography. Dose 1 g. throughout.)

Metabolite	<i>o</i> -Formamidophenol	Benzoxazole	2-Methylbenzoxazole
<i>o</i> -Aminophenol	(2)	(1)	(1)
<i>o</i> -Formamidophenol }	8 { (5-10) (1)	17 { (5-10) (2)	15 { (0) (6)
<i>o</i> -Acetamidophenol }			
<i>o</i> -Aminophenylsulphate	9	3	3
<i>o</i> -Formamidophenylsulphate and/or <i>o</i> -acetamidophenylsulphate	7	13	13
<i>o</i> -Aminophenylglucuronide	52	36	7
<i>o</i> -Formamidophenylglucuronide and/or <i>o</i> -acetamidophenylglucuronide	22	14	28
Hydroxylated benzoxazole†	—	14	32
Hydroxybenzoxazolone	—	4* (3)	—

* Unconjugated. Found by isolation. This value is additional to the total (98%) found by the Ehrlich method.

† This value is the difference between that obtained by the naphthoresorcinol and that by the diazo method.

o-formamidophenol urines. The quantitative results are summarized in Table 5. The amounts of the various metabolites calculated from these are given in Table 6. The difference between the glucuronide values determined by the two methods suggests that about 14% of the dose is excreted as a glucuronide other than that of *o*-aminophenol.

Benzoxazole yields *o*-aminophenol under the conditions for complete hydrolysis of conjugates, so that any unchanged benzoxazole excreted will be included in the estimation of the total amino compounds by the Ehrlich method on completely hydrolysed urines. Since the characteristic smell of benzoxazole was not observed in any of the urines or fractions obtained from them it is probable that the amount of benzoxazole excreted, if any, is very small. There is also the possibility of oxidation of the benzoxazole molecule without rupture of the oxazole ring. This might give benzoxazolone, hydroxybenzoxazole or hydroxybenzoxazolone. Of these only hydroxybenzoxazole would be likely to be estimated along with the total amino compounds (Ehrlich method) and would account for the glucuronide value by the naphthoresorcinol method being larger than the diazo value after mild hydrolysis. Benzoxazolone is not broken down under the

conditions of hydrolysis. The phenolic compounds, if conjugated with sulphuric acid, would be included in the ethereal sulphate excreted.

Two-dimensional paper chromatograms from ether extracts of the urines as collected revealed the presence of *o*-aminophenol, *o*-formamidophenol, *o*-acetamidophenol and a hydroxybenzoxazolone identical with that obtained as a metabolite of benzoxazolone (see below, p. 76). The approximate amounts present were 1, 5-10, 2 and 3% of the dose respectively.

Isolation of metabolites. The only metabolite isolated from ether extracts of unhydrolysed benzoxazole urines was a hydroxybenzoxazolone which was obtained as a pale-brown powder, yield 4% of dose, identical with the compound obtained from benzoxazolone urines. (Found: N, 9.1. Calc. for C₇H₅O₃N: N, 9.3%) *o*-Aminophenylglucuronide, identical with that obtained from *o*-aminophenol, was isolated from the urine by the usual lead procedure. (Yield 12% of dose.)

Metabolism of 2-methylbenzoxazole

If the oxazole ring of 2-methylbenzoxazole is broken in the rabbit as is that of benzoxazole, the metabolites expected would be *o*-acetamidophenol

and *o*-aminophenol and their conjugates. The results of the quantitative analysis of the urine from rabbits which had received 2-methylbenzoxazole are summarized in Table 5 and the amounts of the various metabolites calculated from these data are given in Table 6. 2-Methylbenzoxazole is hydrolysed under the conditions for complete hydrolysis of conjugates. The difference between the glucuronide value and that for the diazo after mild hydrolysis suggests that about 32% of the dose is excreted as a hydroxylated methylbenzoxazole. No evidence for the excretion of unchanged 2-methylbenzoxazole, which has a stronger smell than has benzoxazole, was obtained.

Two-dimensional paper chromatograms from ether extracts of the urines showed the presence of *o*-aminophenol and *o*-acetamidophenol in amounts of approximately 1 and 6% of the dose respectively and a small amount of an unidentified metabolite. The unidentified metabolite reacted with Folin & Ciocalteu's reagent and gave a purple spot on the chromatogram when treated with diazotized *p*-nitraniline. *o*-Hydroxyphenylurea, which has the same R_f in solvent mixtures *A* and *B* (Table 1), gives a mauve spot and has a different R_f in solvent mixture *C*. The difficulty of synthesizing hydroxymethylbenzoxazoles made it impracticable to compare such compounds with the unknown.

Isolation of metabolites. The only metabolite isolated from ether extracts of unhydrolysed 2-methylbenzoxazole urine was *o*-acetamidophenol. Yield 3% of dose. No crystalline glucuronide could be isolated by the usual lead procedure. From hydrolysed urine only *o*-aminophenol was isolated (15% of dose).

Metabolism of 2-phenylbenzoxazole

Preliminary experiments failed to reveal the presence of amino compounds (free or conjugated) in the urine excreted after administration of 2-phenylbenzoxazole to rabbits. This compound is apparently not broken down in the rabbit. The urines were examined quantitatively for ether-soluble acid, phenols, ether-type glucuronide, ethereal sulphate and mercapturic acid. The results obtained are summarized in Table 7. Paper chromatograms from ether extracts of the urines only revealed one phenolic compound in addition to those found in normal rabbit urine. This was the hydroxy-2-phenylbenzoxazole described below. The results suggest that this phenol is excreted free (2% of dose) and conjugated with sulphuric (10%) and glucuronic (48%) acids. The glucuronide is ether-soluble.

Isolation of metabolites. The pooled 24 hr. urines of eight rabbits which had each received 2-phenylbenzoxazole (1 g.) were adjusted to pH 7 and continuously extracted with ether for 48 hr. From the extract 400 mg. of colourless needles, m.p. 215–217°,

were isolated. These gave a positive reaction with Folin & Ciocalteu's reagent but no colour with ferric chloride. A further 120 mg. were obtained by extracting the residual urine with ether at pH 10

Table 7. Results of quantitative analysis of urines from rabbits dosed with 2-phenylbenzoxazole and benzoxazolone

(Results are expressed as average percentage of the dose; ranges in parentheses. Superior figures indicate number of experiments. Dose 1 g. throughout.)

Method of estimation	2-Phenylbenzoxazole	Benzoxazolone
Free phenols	2 (0–4) ^{2*}	2 (1–4) ^{5†}
Conjugated phenols	—	75 (68–86) ^{6†}
Ether glucuronide	48 (41–62) ³	28 (11–43) ^{6†}
Ethereal sulphate	10 ²	22 (21–23) ²
Ether-soluble acid	46 (39–55) ⁶	—
Mercapturic acid	2 (0–4) ^{2§}	2 (0–3) ^{2§}

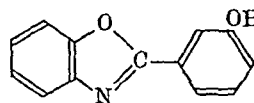
* 2-(3-Hydroxyphenyl)-benzoxazole used as standard.

† Hydroxybenzoxazolone used as standard.

‡ Unreliable since benzoxazolone is a substance which causes excretion of large amounts of reducing material thus giving abnormally high baseline (see Bray, Hybs & Thorpe, 1951).

§ Probably not significant in view of variation in values in normal urine.

(total yield 6% of dose). Some of the compound was boiled under reflux with 10*N*-hydrochloric acid for 2 hr. The reaction mixture was neutralized and continuously extracted with ether. The extract was examined on two-dimensional paper chromatograms using solvent mixture *A* and then *B* (Table 1). Resorcinol, *m*-hydroxybenzoic acid and *o*-aminophenol were identified. This suggests that the compound isolated was 2-(3-hydroxyphenyl)-benzoxazole (VII). (Found: C, 73.5; H, 4.1; N, 6.9. $C_{13}H_9O_2N$ requires C, 73.9; H, 4.3; N, 6.6%.)



(VII)

The residual urine after the separation of the hydroxyphenylbenzoxazole was adjusted to pH 3 and further extracted with ether. The extract was evaporated to dryness and the residue recrystallized from water. Yield 1.3 g., colourless needles, m.p. 202°, which gave a positive naphthoresorcinol reaction. The properties were consistent with those expected of 3-(2-benzoxazolyl)-phenyl glucuronide monohydrate. (Found: C, 55.8; H, 4.6; N, 3.9. $C_{19}H_{17}O_8N \cdot H_2O$ requires C, 56.3; H, 4.7; N, 3.5%. Equiv. 405.) Acid hydrolysis gave resorcinol, *m*-hydroxybenzoic acid and *o*-aminophenol as with the free phenol.

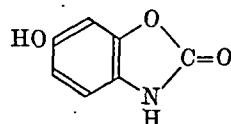
Metabolism of benzoxazolone

This compound is relatively stable to hydrolysis by acids, and the oxazolone ring is not broken under the conditions for complete hydrolysis of conjugates. No evidence of its breakdown *in vivo* was obtained. Urines from rabbits dosed with benzoxazolone were examined quantitatively like those obtained after dosing with 2-phenylbenzoxazole. The results are summarized in Table 7. Only one metabolite of benzoxazolone, a hydroxybenzoxazolone, was detected in ether extracts of the urine by means of paper chromatography. The results suggest that this phenol is excreted free (2 % of dose) and conjugated with sulphuric acid (22 %) and glucuronic acid (53 %). The last figure is derived from the conjugated phenol value since direct determination of glucuronide is unreliable (see footnote to Table 7).

Isolation of metabolites. Continuous extraction with ether of the urine as collected gave a brown powder which separated from the ethereal solution during the extraction. The same substance was obtained by extraction of acidified urine or urine which had been hydrolysed with acid. Yield, 2 g. from 8 g. of benzoxazolone. After crystallization from 50 % (v/v) aqueous ethanol it formed very small pale-brown plates, m.p. 297–298° (decomp. with previous blackening). (Gressly & Nencki, 1890, 1891, stated that the substance blackened at 265°.) The substance gave a positive Folin & Ciocalteu reaction, a green colour with ferric chloride changing to brown, and a purple precipitate with Millon's reagent in the cold (unchanged on boiling). These properties are in agreement with those described by Gressly & Nencki (1890, 1891) for their hydroxybenzoxazolone. (Found: C, 55.9; H, 3.5; N, 9.4. Calc. for $C_7H_5O_3N$: C, 55.6; H, 3.3; N, 9.3 %.) A *glucuronide* (yield 12 % of dose) was isolated from the urine by the usual lead procedure in the form of colourless needles, m.p. 193° (decomp.); $[\alpha]_D^{20} - 79^\circ$ in water (c, 0.5). (Found: C, 43.5; H, 4.5; N, 3.9. Equiv. by reduction, 358; by titration, 336. $C_{13}H_{13}O_9N \cdot 2H_2O$ requires C, 43.0; H, 4.7; N, 3.9 %. Equiv. 363.)

Hydrolysis of the hydroxybenzoxazolone by boiling with concentrated sulphuric acid for 3 hr. or heating in a sealed tube with ammonia (sp.gr. 0.880) at 100° for 3 hr. gave a solution which strongly reduced ammoniacal silver nitrate and gave positive Ehrlich and indophenol tests. (For conditions used for the latter see Thorpe, Williams & Shelswell, 1941.) The properties would be consistent with those of a dihydroxyaniline, but neither this compound nor a derivative could be separated for characterization. A positive indophenol reaction is usually indicative of an —OH group in a position *para* to an —NH₂ group. This admittedly slender

evidence suggests that the —OH in hydroxybenzoxazolone is in position 7 (VIII).



(VIII)

An attempt at synthesis of compound VIII from 2:4-dihydroxybenzamide by treatment with sodium hypochlorite (cf. preparation of benzoxazolone from salicylamide, Graebe & Rostovzoff, 1902; Thorpe & Williams, 1941) resulted in the isolation of colourless needles, m.p. 223–226°. (No blue-green spot characteristic of the hydroxybenzoxazole (Table 1) was detected on paper chromatograms.) The compound gave a purple spot on paper chromatograms and a crimson colour with $FeCl_3$ and was identified as a *chloro-2:4-dihydroxybenzamide*. (Found: C, 44.3; H, 3.2; N, 7.5; Cl, 18.9. $C_7H_5O_3NCl$ requires C, 44.8; H, 3.2; N, 7.5; Cl, 18.9 %.) This amide was very resistant to hydrolysis by hot acid or alkali (sulphuric acid, 80 % (v/v); hydrochloric acid, 10N; phosphoric acid (anhydrous) at 150°; or sodium hydroxide, 40 %, w/v). The formation of an acid from the amide was, however, achieved by Bouveault's (1893) method with nitrous acid although this involved nitration. The chloro-2:4-dihydroxynitrobenzoic acid formed pale-yellow needles, m.p. 242° (decomp.), and gave a blood-red colour with ferric chloride. (Found: N, 6.5; Cl, 16.0. $C_7H_4O_6NCl$ requires N, 6.0; Cl, 15.2 %.) Attempts to prepare this acid by a similar route starting with 2:4-dihydroxybenzoic acid resulted in decarboxylation by the sodium hypochlorite.

Treatment of the hydroxybenzoxazolone from urine with diazomethane gave a *methoxybenzoxazolone*, colourless plates, m.p. 106–107°. (Found: N, 8.6; OMe, 19.2. $C_8H_7O_3N$ requires N, 8.5; OMe, 18.8 %.) No hydroxymethoxyaniline could be isolated after hydrolysis of this compound.

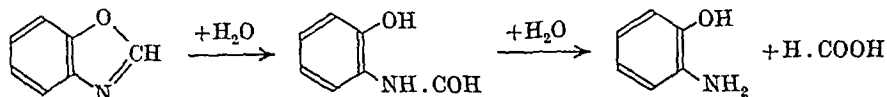
DISCUSSION

The results obtained with the aminophenols are essentially in agreement with those reported by Williams (1938, 1943) and Smith & Williams (1949). In view, however, of some recent observations made in this laboratory upon the conjugation of phenols in the rabbit it is not to be expected that close agreement in quantitative results would be obtained. In that investigation (Bray & White, 1951) it was shown that the percentage of a dose of phenol excreted as ethereal sulphate depends not only upon the available sulphate precursors in the diet and the dose level, but also upon the relation of the time at which the food was consumed to that of dosing. It is,

therefore, unlikely that results obtained in two laboratories will be strictly comparable unless the experimental conditions are very accurately reproduced.

It is clear that in benzoxazole and 2-methylbenzoxazole the oxazole ring may be regarded as a potential centre for conjugation, since the ring is opened and metabolites including *o*-aminophenol are excreted. The results of quantitative analyses suggested the formation also of conjugated hydroxylation products without rupture of the oxazole ring. This is in addition to the small amount of hydroxybenzoxazolone which was isolated from the benzoxazole urine as an unconjugated metabolite and may have been formed by oxidation of a hydroxybenzoxazole.

The formation of *o*-formamidophenol and *o*-aminophenol from benzoxazole suggests that cleavage of the oxazole ring occurs at the C—O linkage. Omitting intermediate stages this could be represented by:



The *o*-acetamidophenol detected was presumably formed by acetylation of the *o*-aminophenol. Attempts to differentiate quantitatively between *o*-formamidophenol and *o*-acetamidophenol in conjugated form by determination of the formic and acetic acids in hydrolysed urines gave unsatisfactory results. It should, perhaps, be mentioned that *o*-formamidophenol was never detected in urines of rabbits which had been given either *o*-aminophenol or *o*-acetamidophenol.

The intermediate expected from 2-methylbenzoxazole would be *o*-acetamidophenol which was actually found in greater amount than from benzoxazole. No *o*-formamidophenol was detected.

In 2-phenylbenzoxazole and benzoxazolone, however, the oxazole ring is stable and no *o*-aminophenol is formed. A new centre for conjugation is formed by hydroxylation. The finding that a considerable percentage of a dose of 2-phenylbenzoxazole or benzoxazolone is hydroxylated is not, therefore, unexpected. The position taken up by the new hydroxyl group introduced is of interest. In 2-phenylbenzoxazole the phenyl group in position 2 is hydroxylated, whereas in benzoxazolone hydroxylation can only occur in the benzene ring fused to the oxazole ring. It would appear that hydroxylation occurs more readily on the ring which is not fused to the oxazole ring, since no evidence of hydroxylation of the benzoxazole moiety of 2-phenylbenzoxazole was obtained.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-aminophenol, *o*-formamidophenol, benzoxazole, 2-methyl- and 2-phenylbenzoxazole and benzoxazolone in the rabbit has been studied.

2. The *o*-, *m*- and *p*-aminophenols were excreted unchanged to the extent of 11, 0 and 2 %; they were excreted conjugated with acetic acid 17, 39 and 45 %, with sulphuric acid 15, 15 and 12 % and with glucuronic acid 65, 64 and 61 % of the dose respectively. The properties of the glucuronides isolated were not in complete agreement with those recorded in the literature.

3. *o*-Formamidophenol was excreted as *o*-aminophenol to the extent of 2 % of the dose, unchanged 7 %, acylated 37 %, conjugated with sulphuric acid 16 %, and conjugated with glucuronic acid 74 %.

4. Benzoxazole was excreted as *o*-aminophenol to the extent of 1 % of the dose, as acylated aminophenol 44 %, as aminophenol conjugated with sulphuric acid 16 %, and as aminophenol conjugated

with glucuronic acid 50 %. About 14 % of the dose was excreted as undiazotizable glucuronide, i.e. hydroxylated without rupture of the oxazole ring. A hydroxybenzoxazolone (4 % of dose) was isolated from urine.

5. 2-Methylbenzoxazole was excreted as *o*-aminophenol to the extent of 1 % of the dose and as aminophenol conjugated with acetic acid 56 %, with sulphuric acid 16 % and with glucuronic acid 35 %. About 32 % of the dose was excreted as undiazotizable glucuronide, i.e. hydroxylated without rupture of the oxazole ring.

6. 2-Phenylbenzoxazole was excreted as free phenols to the extent of 2 %, and conjugated with sulphuric acid 10 % and with glucuronic acid 48 % of the dose. There was no evidence of fission of the oxazole ring. A metabolite believed to be 2-(3-hydroxyphenyl)-benzoxazole and its glucuronide were isolated.

7. Benzoxazolone was excreted as free phenols to the extent of 2 %, and conjugated with sulphuric acid 22 % and with glucuronic acid 53 % of the dose. There was no evidence of fission of the oxazolone ring. A hydroxybenzoxazolone was isolated from urine and its constitution is discussed.

8. R_f values on paper chromatograms for the phenolic metabolites are recorded.

The naphthoresorcinol glucuronic acid determinations were carried out by Miss B. G. Humphris. The microanalyses were carried out by Weiler and Strauss, Oxford.

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Biochemistry of Organic Phosphorus Insecticides

1. THE MAMMALIAN METABOLISM OF BIS(DIMETHYLAMINO)PHOSPHONOUS ANHYDRIDE (SCHRADAN)

BY J. E. GARDINER AND B. A. KILBY
Department of Biochemistry, University of Leeds

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Since the original discovery in 1940 of the high toxicity and powerful anti-cholinesterase activity of dialkyl fluorophosphonates (Adrian, Feldberg & Kilby, 1947), many related organic phosphorus compounds (such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates) have been shown to have similar properties. Certain of these compounds are now being manufactured for use as insecticides, including bis(dimethylamino)phosphonous anhydride, $(\text{NMe}_2)_2\text{P}=\text{O} \cdot \text{O} \cdot \text{P}(\text{NMe}_2)_2$, which is distributed in this country under the trade name of 'Pestox III', and for which the trivial non-proprietary name of 'Schradan' has been recommended, in recognition of the work of Gerhard Schrader who discovered it (Schrader, 1947, 1951). Schradan has unusual properties as an insecticide, in that it shows only weak contact insecticidal action and no fumigant effect, but is readily absorbed into plants through their roots or leaves when applied as an aqueous solution and is transported around the plant in the sap stream. The whole plant is rendered toxic for several weeks to insects, especially aphides, which may feed upon it (Ripper, Greenslade & Lickerish, 1949).

While the mode of action of organic phosphorus compounds as insecticides is still uncertain, there does appear to be some correlation between high insecticidal action and high potency as inhibitors of insect cholinesterases *in vitro* (Metcalf & March, 1949). Schradan is anomalous in that its inhibitory action on this enzyme is only about a millionth of that of some of the more potent phosphorus insecticides; moreover, its inhibition of mammalian cholinesterases *in vitro* is of a similar low order, but if injected into animals it leads to the development of symptoms similar to those following administration of well known anti-cholinesterase drugs and characteristic of acetylcholine poisoning, although the development of symptoms following Schradan is rather slower and death may be delayed a few hours. There appeared to be the possibility that this compound was being converted in the mammalian body into some much more powerful anti-cholinesterase. A greater knowledge of the mammalian metabolism of Schradan is also desirable in view of the possible health hazards which might arise from the accidental consumption by man or animals of plants which had been treated with the insecticide

and still contained appreciable amounts in the sap. We have, therefore, investigated the mammalian metabolism of Schradan using radioactive material, and a preliminary communication has been made (Gardiner & Kilby, 1950a).

MATERIALS AND METHODS

Bis(dimethylamino)phosphonous anhydride (Schradan)

(a) *Non-radioactive.* This was prepared by the interaction of equimolecular amounts of bis(dimethylamino)chlorophosphine oxide, $(\text{NMe}_2)_2\text{POCl}$, (I), with ethyl bis(dimethylamino)phosphite, $(\text{NMe}_2)_2\text{PO}(\text{OEt})$, (II), as previously described (Gardiner & Kilby, 1950b), but with the following modifications. Compound (I) was freed from traces of dimethylamine hydrochloride, $\text{NHMe}_2 \cdot \text{HCl}$, by continuous extraction into petrol (b.p. 40–60°) in which the hydrochloride is insoluble. After removal of the petrol under reduced pressure, (I) was fractionated *in vacuo*. (I) and (II) were heated without solvent for 12–18 hr. at 145° to yield crude Schradan, $(\text{NMe}_2)_2\text{P}=\text{O} \cdot \text{O} \cdot \text{P}(\text{NMe}_2)_2$, which was purified as described below.

(b) *Radioactive.* $^{32}\text{POCl}_3$ was synthesized and converted into labelled Schradan by the method described earlier (Gardiner & Kilby, 1950b) except that the reaction between $^{32}\text{POCl}_3$ and dimethylamine, NHMe_2 (4 mol.), was carried out in petrol (b.p. below 40°), the amine being generated from the calculated amount of $\text{NHMe}_2 \cdot \text{HCl}$ by aqueous KOH (40%, w/v), and carried in a stream of N_2 through a drying tube of KOH pellets into the reaction vessel. When the reaction was complete, the solution was filtered from the precipitated $\text{NHMe}_2 \cdot \text{HCl}$, the solvent removed and the residual radioactive (I) heated overnight at 145° with an excess of non-radioactive (II). The product was purified as below.

(c) *Purification.* The crude reaction mixture containing Schradan was dissolved in aqueous NaOH (0.5% w/v) to destroy unreacted (I) and continuously extracted with benzene for 2 hr. to remove unreacted (II). The aqueous layer was separated and made more alkaline with NaOH (1 ml. 40% (w/v) for each 10 ml. of solution) and the Schradan was then continuously extracted into CHCl_3 . The residue, after removal of CHCl_3 , was fractionated; usually nothing distilled below 100°/0.005 mm. and the pure product (b.p. 102–4°/0.005 mm.) was collected into weighed ampoules, sealed and reweighed. A stock solution of Schradan for radioactive assay and incubation studies was made by weighing out the purified material into a dry graduated flask, adding water, adjusting the pH to 8–9 and storing in the cold room.

Acetylcholinesterase

Preparation. A suspension of human erythrocyte stroma was used. Red cells were separated by centrifugation from a red cell concentrate kindly supplied by the Blood Transfusion Service. Packed cells (1.5 l.) were washed three times with NaCl (900 ml., 0.9%, w/v) and then haemolysed by suspension in water (7.5 l.), the pH (measured with a glass electrode) being adjusted to 5.8–5.9 by the dropwise addition of 0.1 N-HCl with vigorous stirring. After standing overnight at 0°, the stroma had settled and most of the haemoglobin solution could be removed by decantation. The precipitate was resuspended in water (5 l.) and when it had again

settled, the clear solution was removed and the remainder of the preparation spun down to give a cake of cell remains. This was washed a further three times with water (2.3 l.), once with buffer and finally suspended in buffer. When intended for use in manometry, the cell remains were suspended in 600 ml. of buffer containing 0.15 M-NaCl, 0.04 M- MgCl_2 and 0.025 M- NaHCO_3 and saturated with 5% CO_2 –95% N_2 gas mixture. For use in the colorimetric assay, the buffer (600 ml., pH 8.0) contained 429 ml. 0.1 M-sodium diethylbarbiturate and 171 ml. 0.1 N-HCl.

Manometric estimation of cholinesterase. The procedure was essentially that of Ammon (1930), as modified by Nachmansohn (1945), in which the CO_2 production was measured in Warburg manometers at 37°. The main compartment of the cups contained the enzyme, inhibitor and buffer solutions to a total volume of 3 ml. A suitable activity was usually given by 1 ml. of diluted (1 to 50) stock enzyme preparation. The side arm contained acetylcholine bromide (0.2 ml. of 0.1 M stock solution prepared by mixing acetylcholine bromide (1.13 g.), 0.2 M-acetate buffer (pH 4.5, 5 ml.) and water to 50 ml.). The manometers were filled with 95% N_2 and 5% CO_2 by an evacuation procedure (Umbreit, Burris & Stauffer, 1945). The inhibitor and enzyme were mixed for 90 min. before tipping in the substrate. The manometers were equilibrated for 15 min. Normally three readings with an interval of 10 min. were taken before tipping, and four readings afterwards at 10 min. intervals, and the rate of CO_2 output calculated by the statistical procedure described by Aldridge, Berry & Davies (1949). When required, samples were taken from the flasks at the end for radioactive assay.

Colorimetric estimation of cholinesterase. This was developed from the colorimetric method for acetylcholine estimation described by Hestrin (1949), and consisted of measurement of the amount of acetylcholine remaining after the enzyme had been allowed to act for a fixed time. While the method was not as accurate as the manometric one, it was found to be very convenient for the rapid assay of samples. Reagents required were: *Enzyme preparation:* the stock enzyme preparation (1 ml.) was diluted with barbiturate buffer (19 ml., pH 8.0). *Hydroxylamine reagent:* equal volumes of 3.5 N-NaOH and 2 M- $\text{NH}_2\text{OH} \cdot \text{HCl}$ (Analar) were mixed not more than 3 hr. before use. *Ferric chloride:* 0.37 M- $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Analar) in 0.1 N-HCl. *Acetylcholine bromide:* stock solution as described above (2 ml.) diluted with water (5 ml.). *Hydrochloric acid:* conc. HCl was diluted with about twice its volume of water so that the mixture used for colour measurement had pH 1 ± 0.2.

Procedure. The assay was carried out in 6 × 1 in. boiling tubes which could be shaken mechanically in a water bath at 37°. Into each of two tubes were placed 0.6 ml. enzyme solution and 0.3 ml. of water, buffer or appropriate blank solution. One tube was shaken at 37° for 30 min., and then 0.1 ml. acetylcholine bromide solution added rapidly from an all-glass syringe. After exactly 10 min. the enzymic reaction was stopped and the remaining acetylcholine 'fixed' by the addition of 2 ml. of hydroxylamine reagent. To the other tube were added 2.0 ml. of hydroxylamine reagent followed by 0.1 ml. of acetylcholine solution, in order to obtain a measure of the initial concentration of substrate. The two tubes were left at least 1 min. (or up to 5 hr.) and then treated with 1 ml. HCl solution and 1 ml. FeCl_3 solution to form the coloured ferric acethydroxamic acid complex. The solutions were rapidly filtered and the optical densities measured at 5400 Å. on a Unicam D.G.

spectrophotometer. The assay was done in duplicate and the mean difference in optical density between the two pairs of tubes was taken as a measure of the cholinesterase activity of the enzyme. When the potency of a cholinesterase inhibitor solution was being measured, the procedure above was followed except that 0.6 ml. of enzyme and 0.3 ml. of inhibitor solution were used. It was shown that the colour difference was proportional to the amount of enzyme present.

Radioactivity assay

Most measurements were made on solutions using a Geiger-Müller liquid counter of the type described by Veall (1948) and made by 20th Century Electronics Ltd. All assays were made using the 'counter full' conditions, and the concentration of active material in a sample estimated by comparison of count with that of a solution of active Schradan of known molarity. After correction for background, the counting rate for a given solution was converted back to what it would have been at some arbitrary zero time. This time was chosen as that when the standard solution gave a convenient rate, e.g. 100 counts/min. for the counter filled with 10^{-5} M solution.

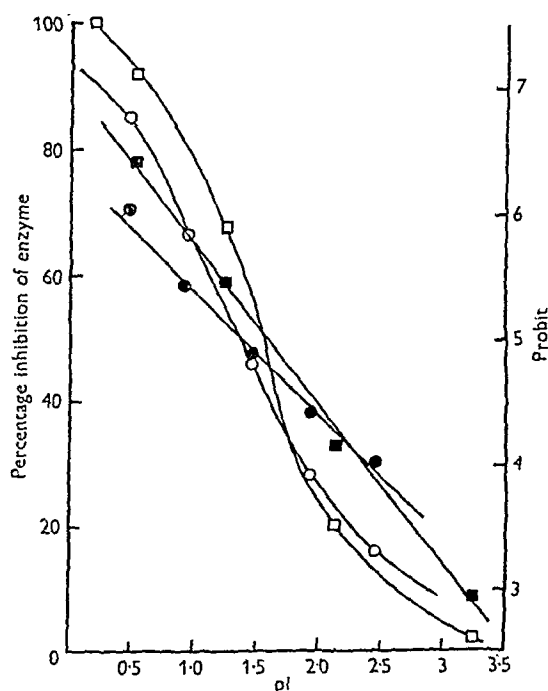


Fig. 1. Inhibition of cholinesterases by Schradan. Whole rabbit blood cholinesterase: \square , percentage inhibition; \blacksquare , probit. Human erythrocyte stroma cholinesterase: \circ , percentage inhibition; \bullet , probit. pI is the negative log molar concentration of inhibitor.

Some of the assays on blood were carried out by withdrawing 0.1 ml. blood with a blood pipette and absorbing on small disks of filter paper fitted into inverted standard nickel sample trays. The pipette was washed out with 0.1 ml. of saline. The disks were then counted under a General Electric Company Type G.M. 4 end-window counter. The standard for comparison was 0.1 ml. of solution of known molarity assayed in the same manner.

RESULTS

Inhibitory action of Schradan on cholinesterases

The percentage inhibition of the cholinesterases in whole rabbit blood and in the human erythrocyte stroma preparation, following incubation for 90 min. with different concentrations of pure Schradan, was measured manometrically. Controls were made to correct for carbon dioxide produced by spontaneous hydrolysis of the substrate and, in the case of rabbit blood, by cell respiration. The percentage inhibition/log concentration curves were of the usual S-type, and after the probit transformation (Finney, 1947) give linear regression lines (Fig. 1). The concentrations for 50% inhibition are given in Table 1. The low inhibitory action of Schradan

Table 1. Inhibition of cholinesterases by Schradan

Source of cholinesterase	Concn. of Schradan for 50% inhibition (M)
Whole rabbit blood	2.6×10^{-2}
Human erythrocyte stroma	4.5×10^{-2}
Bee brain (Metcalf & March, 1949)	$>1.2 \times 10^{-3}$

compared with the high potency of other compounds which are sometimes present as impurities in ordinary samples of Schradan, necessitate a careful purification of the compound before assay.

In vivo inhibition of rabbit blood cholinesterase

A blood sample (5 ml.) was taken from a rabbit, either from an ear vein or by heart puncture. The animal was then given an intraperitoneal injection of radioactive Schradan (50 mg./kg. in mammalian Ringer solution, this dose being approx. $5 \times \text{LD}_{50}$). Blood samples (0.1 ml.) were subsequently withdrawn from the ear, initially every 5–10 min. and then at longer intervals until the death of the animal when a final blood sample of 5–10 ml. was taken from the heart. Death occurred 2–6 hr. after injection, and was preceded by the familiar symptoms of acetylcholine poisoning seen after the administration of anti-cholinesterase drugs (excessive salivation, fibrillary twitchings, defaecation, muscular weakness and convulsions leading to death).

Radioactive assays were made on the blood samples (end-window counter technique) and the apparent concentration of Schradan present in the blood was calculated on the assumption that all the radioactivity observed was due to this compound; results from two rabbits are shown in Fig. 2. The maximum concentration attained was not essentially different from that observed at death. The cholinesterase activity of the blood at death was compared by the manometric method with that of

blood taken before injection, and results from a number of experiments are given in Table 2. The mean concentrations of Schradan necessary *in vitro* to produce the inhibition observed at death, as

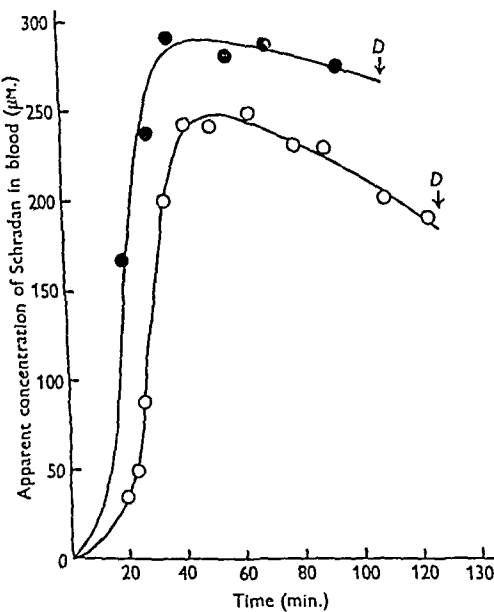


Fig. 2. Apparent concentration of Schradan in the blood of two rabbits following intraperitoneal injections of 50 mg. radioactive Schradan/kg. (D, death of animal.)

given by the curve in Fig. 1, have also been entered in Table 2. In some experiments, Schradan was added to blood withdrawn before injection at the same time as the rabbit was injected, and the mixture incubated at 37° until the animal died, enabling a comparison to be made of the *in vivo* and *in vitro* inhibition on the same blood (Table 3). A considerable enhancement of activity *in vivo* has occurred.

In one experiment, a rabbit was injected with 50 mg./kg. radioactive Schradan and blood samples (1 ml.) were withdrawn at intervals until death. Radioassays and cholinesterase activity measurements by the manometric method were made on each sample. As the concentration of radioactive material in the blood increased, the cholinesterase activity fell, until at death (after 222 min.) the apparent Schradan concentration was $8.8 \times 10^{-5}M$ and the cholinesterase was 93 % inhibited. There was some doubt as to whether the injection had been intraperitoneal (as intended) or subcutaneous; injection by the latter route may account for symptoms developing rather more slowly than usual and death being delayed.

Incubation of Schradan with liver slices

The purpose of these experiments was to see if there was any evidence of an enhancement of the activity of Schradan following incubation with liver

Table 2. Cholinesterase inhibition and apparent Schradan concentration in post-mortem rabbit blood, following intraperitoneal dose of 50 mg./kg. Results on six animals

Inhibition of cholinesterase in post-mortem blood (%)	Apparent concn. of Schradan in blood by radioassay ($\times 10^{-5}M$)	<i>In vitro</i> concn. of Schradan necessary to give observed inhibition (M)	Enhancement of activity <i>in vivo</i>
98.0	34.7	0.55	1600
95.0	50.4	0.40	790
93.0	8.8	0.33	3750
93.0	7.5	0.33	4500
89.0	4.3	0.24	5700
68.0	1.7	0.074	4400

Table 3. *In vivo* and *in vitro* inhibition of whole rabbit blood cholinesterase by Schradan. Results on two rabbits

<i>In vivo</i> Results from post-mortem blood following dose of 50 mg./kg.			<i>In vitro</i> Schradan added to initial blood		
Apparent concn. of Schradan by radioassay (mM)	Apparent concn. of Schradan during cholinesterase estimation (mM)	Inhibition of cholinesterase (%)	Concn. of Schradan during incubation (mM)	Concn. of Schradan during cholinesterase estimation (mM)	Inhibition of cholinesterase (%)
0.017	0.0029	68	110	44	50
0.043	0.0071	89	50	17.5	29
			0.375	0.125	5
			0.75	0.25	0
			1.50	0.50	2.5

Table 4. *Enhancement of anti-cholinesterase activity of Schradan by incubation with rabbit-liver slices*
(1 g./10 ml. medium for 3 hr.)

Exp. no.	Mixture assayed	CO ₂ production (μl./min.)	Esterase inhibition (%)
A 1	Supernatant from slices incubated in buffer solution (control for 2 and 3)	8.25	—
2	Supernatant from slices incubated with Schradan (3.0×10^{-3} M in buffer)	3.47	57.9
3	Supernatant from slices incubated with Schradan (1.45×10^{-2} M in buffer)	1.89	77.1
4	Buffer solution only (control for 5 and 6)	7.30	—
5	Schradan (3.32×10^{-3} M in buffer solution)	7.21	1.2
6	Schradan (1.55×10^{-2} M in buffer solution)	4.98	31.7
B 7	As (1) above (control for 8)	9.05	—
8	As (2) above, but Schradan 1.93×10^{-2} M	2.15	76.3
9	Buffer solution only (control for 10)	5.62	—
10	Schradan (1.93×10^{-2} M in buffer)	3.35	40.4
C 11	Supernatant from slices incubated with buffer (control)	5.88	—
12	Supernatant from slices incubated with Schradan (final concn. 5.5×10^{-4} M) buffer	3.73	36.6
13	As 12, but Schradan added after incubation	5.52	6.1
14	Supernatant from slices incubated with Schradan (2.8×10^{-4} M)	4.15	29.4
15	As 14, but Schradan added after incubation	5.43	7.6

slices. Rabbit or rat liver was used, the animal being killed by a blow on the head, the liver removed and dropped into ice-cold mammalian Ringer solution, and subsequently sliced by hand and added to the appropriate medium at the rate of 1 g./10 ml. In later experiments the mixture was gently shaken and a stream of air blown over the surface while being incubated at 37°. After a suitable period the mixture was centrifuged and the supernatant fluid kept at 0° until inhibitory activity measurements were made (usually within an hour). The human erythrocyte preparation was used as a source of cholinesterase in the assays unless otherwise stated.

The results of three experiments using rabbit-liver slices from different animals are shown in Table 4. The slices were incubated for 3 hr. and 1 ml. of centrifuged supernatant fluid assayed by the manometric method. The first two experiments were similar in their design, but as it was evident that the supernatant liquid showed some liver esterase activity, the third experiment (C) was arranged so that all solutions assayed contained the same amount of supernatant fluid from the slices, but differed in their treatment, the Schradan being added either before or after incubation and centrifugation of the slices; this comparison was made for two concentrations of Schradan. Rat-liver slices were used in experiments D, and cholinesterase assay was carried out by the colorimetric method. The recorded colour value is proportional to the amount of acetylcholine split in 10 min. The glucose buffer used was Krebs' Ringer phosphate (pH 7.4), containing 0.01 M-glucose. Results are shown in Table 5.

Table 5. *Enhancement of anti-cholinesterase activity of Schradan by incubation with rat-liver slices*

(1 g./10 ml. medium for 3 hr.)			
Exp. no.	Mixture assayed	Colour difference	Esterase inhibition (%)
D 16	Supernatant from slices incubated in glucose buffer	0.38	—
17	Supernatant from slices incubated in glucose buffer containing Schradan (1.3×10^{-3} M)	0.07	81
18	As (17), but without glucose	0.08	79
19	As (18), but Schradan added after incubation	0.38	0

Stability

The supernatant liquid from rat-liver slices incubated with 1.2×10^{-3} M-Schradan in buffer gave 81% inhibition of cholinesterase initially, but no inhibition after heating for 10 min. at 100°. In a similar experiment, using 1.91×10^{-3} M-Schradan in glucose buffer, the inhibition fell from 87 to 3% after the same treatment. The active material was thus destroyed by this treatment, which was shown to have little or no effect on Schradan itself. The active material was more stable at 0°, as three different samples of supernatants which inhibited the enzyme by 97, 87 and 81% initially, after keeping for 24 hr. at 0°, gave inhibitions of 86, 65 and 73% respectively.

Dialysis

It was considered that further investigation of the properties of the active material would be facilitated if it could be obtained in a protein-free solution. An active extract was enclosed in a cellophan bag and dialysed against an equal volume of buffer for 18 hr. and assays of inhibition made by the colorimetric method (Table 6). The active material had passed

supernatants dialysed (Table 7). The cellophan used was soaked in water before use in order to remove traces of soluble material which were found to have an inhibitory action on cholinesterase.

Extractability

The active material can be obtained in a salt- and protein-free aqueous solution by extraction of an active dialysed liver-slice extract with chloroform, removal of the solvent and solution of the residue in water. In a typical experiment an active dialysate (30 ml.) which gave almost complete inhibition of cholinesterase and which contained 85.8 μ g. atoms P (by radioactivity assay) was extracted with chloroform (5 \times 18 ml.). The extracted solution had almost entirely lost its inhibitory activity and the content of material had dropped to 3.2 μ g. atoms P. The chloroform was removed from the extract under reduced pressure at room temperature and the residue dissolved in water (13.6 ml.); the resulting solution again almost completely inhibited the enzyme and contained 73.2 μ g. atoms P. (Control solutions were treated with chloroform in the same manner.) Other solvents, such as petrol (b.p. under 40°), nitromethane and nitrobenzene were tried, but little or no inhibitory or radioactive material was extracted.

Stability to acid and alkali

through the cellophan, and the dialysate was a powerful inhibitor of cholinesterase although the concentration of active material in it was only half that in the original solution. The slight decrease in activity of the control was probably due to the elimination of the liver esterase activity. A slightly more elaborate experiment was then made in which Schradan was added before and after the incubation and centrifugation of the liver slices, and the two

The effect of exposing the active material for a short time to 0.033N-acid or alkali was investigated. After 5 sec. the mixture was neutralized and the

Table 6. *Dialysis of activated Schradan extracts*

Exp. no.	Solution assayed	Colour difference	Esterase inhibition (%)
20	Supernatant from slices incubated in buffer alone	0.34	—
21	Supernatant from slices incubated with 2.2×10^{-3} M-Schradan in buffer	0.05	85.5
22	Dialysate from (20)	0.31	—
23	Dialysate from (21)	0.07	77.5

Table 7. *Dialysis of activated Schradan*

(Supernatant I: from 4 g. rat-liver slices + 30 ml. buffer + 1.1 ml. water. Supernatant II: from 4 g. rat-liver slices + 30 ml. buffer + 1.1 ml. Schradan (5.9×10^{-2} M).)

Exp. no.	Origin of dialysate assayed	Colour difference	Esterase inhibition (%)	Apparent concn. of Schradan from radioactivity (mM)
20	10 ml. of (I) + 0.4 ml. water against 10.4 ml. buffer (control)	0.34	—	—
21	10 ml. of (I) + 0.4 ml. Schradan (5.9×10^{-2} M) against 10.4 ml. buffer	0.34	0	1.52
22	20 ml. of (II) + 0.8 ml. water against 20.8 ml. buffer	0.005	98.5	0.88

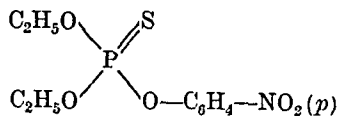
Table 8. *Instability of active material in acid and alkaline solution*

Mixture	5 sec. exposure		30 sec. exposure	
	Colour difference	Esterase inhibition (%)	Colour difference	Esterase inhibition (%)
Water (1 ml.) + 0.1 N-HCl (0.5 ml.) + 0.1 N-NaOH (0.5 ml.)	0.379	—	0.380	—
Active extract (1 ml.) + mixture of 0.1 N-HCl (0.5 ml.) and 0.1 N-NaOH (0.5 ml.) (control)	0.033	91.5	0.100	73.0
Active extract (1 ml.) + 0.1 N-NaOH (0.5 ml.) and later 0.1 N-HCl (0.5 ml.)	0.040	89.4	0.160	57.8
Active extract (1 ml.) + 0.1 N-HCl (0.5 ml.) and later 0.1 N-NaOH (0.5 ml.)	0.334	14.0	0.370	2.5

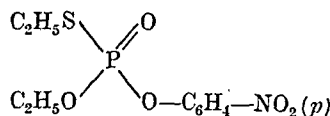
inhibitory power assayed by the colorimetric method. A similar experiment was made using a different extract but delaying neutralization for 30 sec. (Table 8). The active material is seen to be largely destroyed by the alkali within 5 sec., but is slightly more stable towards 0.033*N*-acid.

DISCUSSION

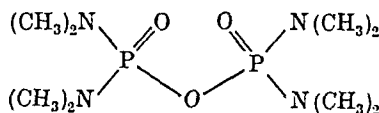
The experimental results show that Schradan has a low *in vitro* anti-cholinesterase activity, but produces a fatal acetylcholine-like poisoning in animals,



III



IV



V

and that the apparent contradiction can be explained by the conversion of the compound in the body into some much more potent inhibitor of the enzyme. The enhancement of activity can also be produced *in vitro* by incubation of the compound with liver slices, suggesting that the liver is involved in the alteration of Schradan in the body. The conversion into a more active poison would offer an explanation of why death is delayed for a few hours after injection of a lethal dose, whereas administration of certain other organic phosphorus anti-cholinesterases which are highly active *in vitro* (such as tetraethylpyrophosphate and dialkyl fluorophosphonates) leads to death within a few minutes. Soon after our preliminary communication (Gardiner & Kilby, 1950*a*) a paper was published by DuBois, Doull & Coon (1950) in which similar findings were made quite independently and similar conclusions reached. Recent work suggests that other organic phosphorus compounds may undergo a similar enhancement of activity *in vivo*. Holmstedt (1951) has shown that a linear relationship between the logarithm of the LD₅₀ and that of the concentration causing 50% inhibition of cholinesterase *in vitro* holds for certain very active organic phosphorus anti-cholinesterases, while for other related compounds, such as Schradan and bis(dimethylamino)-phosphoryl fluoride, (NMe₂)₂POF, the relationship does not hold owing to low *in vitro* activity, and Holmstedt suggests that conversion *in vivo* may be the explanation. Diggle & Gage (1951) consider that the inhibition of brain cholinesterase following injection into rats of highly purified Parathion (III)

is greater than that to be expected from *in vitro* measurements and cannot be ascribed solely to Parathion or possible impurities.

Parathion is isomerized by heat to give (IV), which has been shown by Diggle & Gage to be a thousand times more active *in vitro* than Parathion itself as an anti-cholinesterase, and it is possible that a similar isomerization catalysed *in vivo* is the cause of the enhancement of activity of Parathion when injected. It is difficult to see how Schradan (V) could isomerize and it would appear that the molecule must undergo some other type of chemical

change. In water or alkaline solution, Schradan is hydrolysed very slowly ($k = 4.7 \times 10^{-3} [\text{OH}^-] \text{ min.}^{-1}$ at 100°), but more rapidly in acid solution

$$(k = 3.6 \times 10^{-3} [\text{H}^+] \text{ min.}^{-1} \text{ at } 25^\circ)$$

when the ultimate products of hydrolysis are phosphoric acid and a dimethylamine salt (Hartley & Heath, 1951). These workers, using radioactive Schradan, have shown that the mode of decomposition in plants is wholly different. Schradan would have a half-life *in vitro* of over 8 years at pH 4.5–7.0 (that of plant saps), yet only 10% remained unchanged 4 weeks after absorption of the compound by a plant in vigorous growth, and up to 50% was present as decomposition products. Using a related compound, tris-(dimethylamino)-phosphine oxide (containing no P—O—P linkage), it was shown that the decomposition products containing phosphorus, which were present in plants, unlike those from acidic hydrolysis, were extractable from alkaline solution by chloroform, so that acidic hydroxyl groups on the phosphorus atoms could not be present, and it was suggested that in plants the enzymic attack is on a N—C or C—H and not on a P—O or P—N bond. Plants, therefore, appear to contain enzyme systems capable of metabolizing Schradan, but it is not yet possible to say whether or not the changes produced are similar to those taking place in mammalian tissues.

Evidence is accumulating that the organic phosphorus compounds inhibit enzymes by a type of phosphorylation in which a substituted phosphoric acid residue remains attached to the enzyme

molecule. Most of the organic phosphorus enzyme inhibitors are acid anhydrides in the broad sense (e.g. tetraethylpyrophosphate, dialkyl fluorophosphonates) and contain a bond on the phosphorus atom which is ruptured by the enzyme in a nucleophilic substitution reaction (S_N2 type mechanism) (Wilson & Bergmann, 1950). Studies with chymotrypsin have shown that when this bond is broken, the portion of the molecule containing phosphorus joins on to the enzyme while the other part is liberated as an anion. Thus, when chymotrypsin is inhibited by radioactive diisopropylfluorophosphonate it has been shown (Jansen, Nutting & Balls, 1949; Jansen, Nutting, Jang & Balls, 1950) that one radioactive phosphorus atom and two isopropoxy groups are present in each molecule of inhibited enzyme, and one molecule of acid (presumably HF) is liberated; while Hartley & Kilby (1950) have shown that one molecule of *p*-nitrophenol is liberated for each molecule of chymotrypsin inhibited by the oxygen analogue of Parathion. On this basis, one would expect the active molecule derived from Schradan to contain a bond of the high energy type, making the compound a more powerful phosphorylating agent and thus a more efficient enzyme inhibitor. We consider that the activation process may well be the fission of the P—O bond and the replacement of the —O—P(NMe₂)₂ portion by some group X such that the P—X bond is a labile and reactive one; it might, for example, be an acetate or phosphate group. It cannot be a hydroxyl group resulting from a simple hydrolytic fission of the molecule into two identical halves, as the compound (NMe₂)₂PO(OH) has been examined and is stable and devoid of anti-cholinesterase activity; nor can this be the precursor of the active compound as this is not generated from it by incubation with liver slices. The instability of the

active material in 0.033N-alkali for 5 sec. indicates that a labile molecule has been produced.

The procedure outlined has led to the isolation of a preparation which contains the active material labelled with ³²P and present in a salt and protein-free solution. It is likely that the highly inhibitory material is present only in small amounts in these active preparations, and that the major part of the radioactivity is due to unchanged Schradan. Some of the preparations contained ³²P in concentrations of the order 10⁻²M without giving complete cholinesterase inhibition, whereas the ³²P content of the blood of rabbits at death after poisoning by Schradan, was only about 10⁻⁴–10⁻⁵M, yet the blood cholinesterase was almost completely inhibited. Further investigation on the nature of the active substance is proceeding.

SUMMARY

1. A 50 % inhibition of whole rabbit blood cholinesterase is caused by incubation for 1 hr. with 2.6 × 10⁻²M-Schradan, bis(dimethylamino)phosphonous anhydride. In spite of this low inhibitory action, injection into rabbits leads to death with the symptoms of acetylcholine poisoning.

2. Using radioactive Schradan, it is shown that the compound is converted *in vivo* into some more active anti-cholinesterase.

3. Incubation of Schradan *in vitro* with rat- or rabbit-liver slices leads to a similar enhancement of activity.

4. The active material after liver-slice incubation can be dialysed into buffer and extracted with chloroform; it is labile and is destroyed by 5 sec. exposure to 0.033N-alkali, but is slightly more stable to acid.

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The Role of Ammonia in Ruminal Digestion of Protein

By I. W. McDONALD

Agricultural Research Council, Institute of Animal Physiology, Babraham Hall, Cambridge

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In a previous communication attention was drawn to the occurrence in the rumen of the sheep of significant concentrations of ammonia (McDonald, 1948*a*). It was shown that urea was present in the saliva of sheep, and that this was one source of ammonia, since Lenkeit & Becker (1938) had demonstrated a high urease activity of the micro-organisms of the rumen. Evidence was also presented to prove that ammonia was absorbed from the rumen into the portal blood stream, and, since only mere traces of ammonia could be detected in the blood of the general circulation, it was concluded that the absorbed ammonia was converted by the liver into urea; thus a circulation of nitrogen via the saliva, ruminal ingesta and liver could be envisaged. Further observations which indicate the importance of ammonia in the turnover of nitrogen in the rumen are reported in this paper.

EXPERIMENTAL

In all experiments the sheep were fed once daily, but were allowed continuous access to water. The simple diets used were always adequate to maintain the animals in robust health.

The sheep used in these experiments were provided with rumen fistulae, to enable sampling of the rumen contents. No means are available for taking a representative sample of the rumen contents; this difficulty, which has been discussed by Pearson & Smith (1943), is due to the fact that the rumen contents are heterogeneous and contain large fragments of plant material which are not uniformly distributed throughout the entire mass of ingesta. In order to avoid this difficulty, the analyses recorded here were made on the fluid obtained from the rumen contents after straining through muslin. The object was to obtain a sample of the rumen liquor which would contain the soluble nitrogenous constituents and the micro-organisms, but not the dietary plant fragments. This expedient was not entirely satisfactory, since some of the smallest plant fragments passed through the muslin, while large numbers of micro-organisms were retained with the plant residues. It was, however, observed that samples of rumen liquor obtained from widely separated points in the rumen showed the same composition. It is also probable that the smallest plant fragments were those which had been most thoroughly attacked by the ruminal bacteria and hence contributed but little protein to the mixture. The most unsatisfactory feature was the loss of micro-organisms on the plant residues and thus the reduction of the concentration of protein in the rumen liquor.

The rumen liquor will also contain some protein from the saliva, but the concentration of protein in saliva is small in

comparison with that of the rumen liquor, and since the salivary protein would also be susceptible to digestion in the rumen, any error from this source was presumed to be negligible.

With these reservations, it may be taken that the protein of rumen liquor consists essentially of the protein of the contained micro-organisms. Indirect evidence in favour of this conclusion was found in the very high concentration of protein in the dry matter of the rumen liquor; an average of nine analyses gave the value of about 50% protein (protein N \times 6.25) in the dry matter after allowance was made for the content of ash and volatile fatty acids. These reservations do not, however, preclude the use of analyses of rumen liquor to observe the trend of changes in the distribution of nitrogen in the rumen in relation to time after feeding, and the procedure is certainly valid for the soluble non-protein nitrogen (N.P.N.) constituents; only limited conclusions can be drawn from the values obtained for protein N.

Methods

Total nitrogen was estimated by the Kjeldahl method, using the semi-micro technique of Chibnall, Rees & Williams (1943).

Ammonia was estimated by the method of Conway (1947) or by distillation of protein-free filtrates with NaOH-borate buffer at pH 8.5. The two methods gave excellent agreement and were in accord with values obtained by the method of Parnas & Klisiecki (1926).

Non-protein nitrogen (N.P.N.) was estimated as the total N in protein-free filtrates obtained either by precipitation with ethanol or with dilute acid. It was found that the protein of rumen liquor, obtained by filtering rumen contents through muslin, could be quantitatively precipitated by dilution (1 to 10) and acidification to approximately pH 2.5.

Protein N was calculated as total N minus N.P.N.; for convenience of description, the N.P.N. was divided into two categories, ammonia N and residual N. The components of the residual N have not been studied.

RESULTS

Meadow-hay diet

Preliminary observations were made with sheep fed an exclusive diet of meadow hay; a graph showing the changes in the distribution of nitrogen in the rumen liquor is given in Fig. 1. The values shown are the averages of three observations on each of two sheep. The main features shown by these curves are as follows. The N.P.N. consists chiefly of ammonia N, while the residual N is always of low concentration. Qualitatively, amino-acids could not be detected by direct ninhydrin tests or by paper chromatography of protein-free filtrates. The rapidity with which the

ammonia accumulates in the rumen after feeding is a reflexion of the high activity of the micro-organisms. Since amino-acids do not accumulate in the ingesta, it is apparent that the rate of uptake of amino-acids by the microbes from the medium exceeds the rate of proteolysis by the proteases formed by them, or that they may in addition actively deaminate free amino-acids.

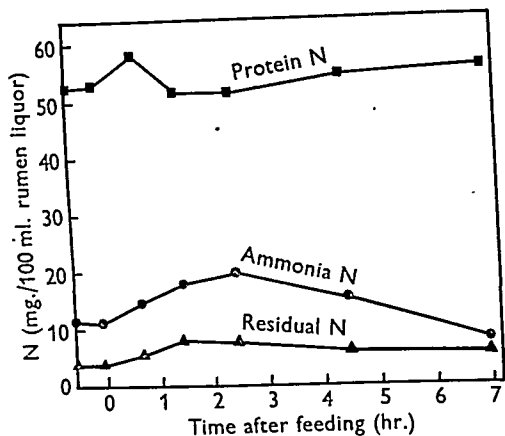


Fig. 1. Changes in the distribution of nitrogen in the rumen liquor of sheep on a diet of meadow hay. Each point represents the average of three observations on each of two sheep.

The peak in the curve for ammonia concentration does not give an indication of the magnitude of total formation of ammonia since this is removed from the rumen in several ways: by its utilization by bacteria for their growth, by passage in the ingesta from the rumen to the omasum and abomasum, and by direct absorption from the rumen (McDonald, 1948a). The relative magnitude of these effects has not yet been estimated with accuracy.

Casein and zein diets

The close association of the changes of concentration of ammonia in the rumen with feeding suggested that part of the ammonia was derived from the proteins of the feed. In order to test this hypothesis, proteins in suspension were added directly to the rumen through a fistula. It was first established that a satisfactory base-line could be obtained by sampling the rumen contents during the period of 16–24 hr. after feeding; the control curve in Fig. 2 shows that during this time the concentration of ammonia in the rumen remains virtually constant; the factors responsible for the accumulation and removal of ammonia have thus come into equilibrium. Casein, gelatin and zein were chosen as representative proteins on the ground that the two former are soluble and readily attacked by proteinases, while the latter is highly insoluble in aqueous media and relatively resistant to proteo-

lysis. The results given in Fig. 2 show clearly that the addition of 25 g. casein to the rumen was followed by a pronounced rise in the ammonia concentration. An approximate estimate indicates that the ammonia nitrogen formed from the casein represented about 20% of the total N added. Since amide N comprises only 9.3% of the total N of casein, the observed rise in ammonia cannot be due solely to the

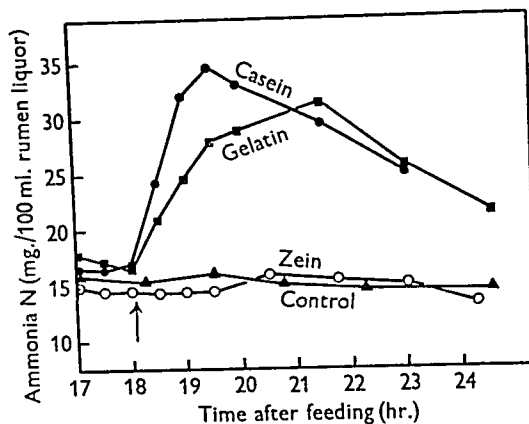


Fig. 2. Comparisons of the effects on ammonia formation of addition of protein suspensions to the rumen of a sheep; 25 g. of protein were added at the time indicated by the arrow. Control curve, no protein added.

removal of amide groups; it was concluded therefore that deamination as well as deamidation reactions were responsible for the formation of ammonia. This conclusion was supported by the fact that similar results were obtained when a solution of gelatin, which contains only a trace of amide N (Chibnall, 1942), was added to the rumen.

The direct addition of 25 g. zein to the rumen produced no change in the concentration of ammonia N in the rumen. This is clearly a reflexion of the physical properties of the protein which render it so resistant to proteolysis (Laine, 1944). Since zein is in fact digested to a considerable extent in the rumen (McDonald, 1948b) it is evident that its rate of digestion is too slow to permit an accumulation of ammonia.

These experiments showed that ammonia could be derived from protein in the feed. Observations were then made on the distribution of nitrogen in the rumen fluid when sheep were fed on partially purified diets in which casein or zein comprised the chief source of nitrogen. The daily ration was composed of protein (casein or zein), 110 g.; starch, 280 g.; cellulose, 250 g.; glucose, 80 g.; molasses, 40 g.; chaffed straw, 150 g. and adjuvants of mineral salts and vitamins A and D; the prepared diets were found to be palatable and the day's ration was consumed within a few hours.

The distribution of nitrogen in the rumen liquor during feeding regimes with these two diets is

shown in Figs. 3 and 4. Again it is clear that the soluble casein is readily attacked with the liberation of ammonia and of other N.P.N. substances (expressed as residual N). The rise in residual N is of comparatively brief duration, and after 2 hr. the

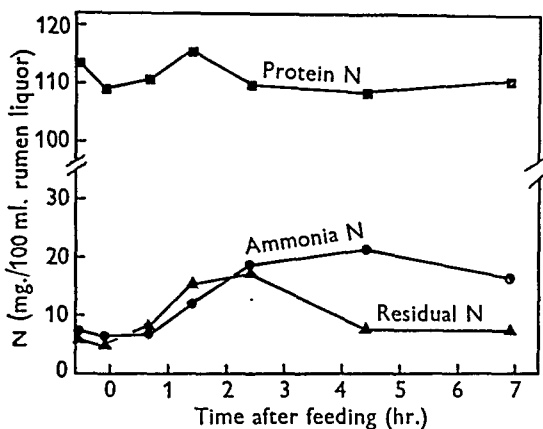


Fig. 3. Changes in the distribution of nitrogen in the rumen liquor of a sheep on a diet in which casein comprised the chief source of nitrogen. Each point represents the average of three observations.

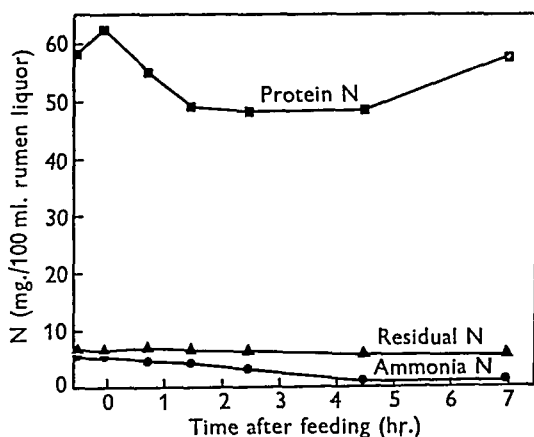


Fig. 4. Changes in the distribution of nitrogen in the rumen liquor of a sheep on a diet in which zein comprised the chief source of nitrogen. Each point represents the average of four observations.

value begins to decline while the level of ammonia N continues to rise for 4 hr. after feeding. In sharp contrast is the effect of feeding zein in the diet; the ammonia falls to extremely low levels after feeding, while the residual N shows no change. This finding can best be interpreted as indicating that the rate of proteolysis of the zein is slower than the capacity of the bacteria to take up the products of proteolysis and that in the presence of readily available sources of energy (in this case, glucose and starch) the organisms use the ammonia as a source of nitrogen for growth; later when the growth rate of the bacteria declines, the rate of formation of ammonia

may exceed the rate of uptake and the concentration of ammonia rises slowly to the pre-feeding level. Further evidence in support of this view is the observation that when starch alone is added to the rumen during the late post-feeding stage there is a steady decline in the concentration of ammonia; the results of an experiment with two sheep are shown in Fig. 5.

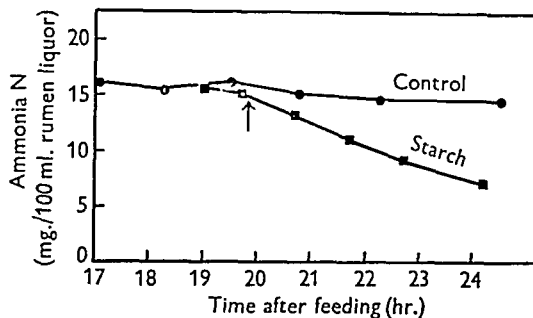


Fig. 5. The influence on ammonia concentration in the rumen liquor of addition of starch suspension to the rumen of sheep during the late post-feeding phase.

The relative availability of the two proteins for bacterial growth is illustrated in the marked difference in the levels of protein N in the rumen liquor in spite of the fact that both diets contained the same amount of nitrogen. On the zein diet, the protein N level in the rumen liquor was approximately 55 mg./100 ml. compared with 110 mg./100 ml. on the casein diet.

DISCUSSION

The general purpose of the work reported here was to obtain information, both qualitative and quantitative, on the role of micro-organisms in the digestion of protein in the rumen. It has already been established by Wegner, Booth, Bohstedt & Hart (1940) that the saliva of ruminants contains no proteolytic enzymes and it is well known that the stratified squamous epithelium of the mucosa of the rumen and reticulum possesses no secretory glands. It is therefore evident that digestion of protein in the rumen can be due only to proteolytic enzymes contained in the food or produced by the microbes (protozoa and bacteria) which inhabit this viscus. In order to eliminate the former as a significant factor, preliminary experiments were conducted with diets consisting exclusively of hay, in which the leaf proteins are denatured by the drying of the leaves (Lugg, 1946); under these conditions it was considered that little, if any, proteolysis could be ascribed to surviving plant enzymes. By contrast, the rumen supports an extremely large population of microbes; Van der Wath & Myburgh (1941) recorded protozoal counts exceeding 2 millions/ml. of rumen contents, and

Gall, Burroughs, Gerlaugh & Edgington (1949) found bacterial counts exceeding 50×10^9 /g. of rumen contents. Since this dense population of microbes is maintained in spite of continuous passage of ingesta from the rumen, it is evident that a significant fraction of the nitrogen of the host's diet must be digested and utilized by the microbes for their own growth. The presence of highly active proteinase, considered to be of microbial origin, in the ruminal contents was demonstrated by Sym (1938), who used casein as substrate for *in vitro* tests. There is little reason to doubt that, for all practical purposes, the whole of the digestion of protein in the rumen is effected by micro-organisms.

In the general physiological economy of the ruminant, the essential function of the rumen may be envisaged as the digestion of cellulose and other carbohydrates for which the animal does not produce digestive enzymes; other changes in the rumen can be considered as coincidental to this function. In this respect, the anatomical arrangement of the digestive organs, which provides for the microbial digestion of cellulose prior to the operation of the animal's own enzymic secretions, confers on the ruminant a more efficient mechanism than is found in other herbivores, in which the bacterial degradation of cellulose follows the activity of the gastric and intestinal secretions. The digestion of cellulose requires the provision by the host animal of an environment in which the cellulose-splitting bacteria may thrive; this environment is provided in the rumen with a high degree of regulation (Phillipson, 1946), with the result that a population of bacteria and protozoa in very large numbers is maintained. This population requires a suitable and adequate supply of nitrogen; under normal feeding conditions, most of the nitrogen in the animal's diet will be comprised of protein, but other nitrogenous substances will always be present. For none of the natural feeds are complete analyses available for the distribution of nitrogenous substances; in some cases it is known that amides or free amino-acids contribute to the N.P.N. fraction, but most natural feeds will contain a variety of N.P.N. substances in other forms.

Since so many organisms are capable of using ammonia as the sole or part source of nitrogen for growth, and since ammonia is also the chief nitrogenous end product in the breakdown of proteins by bacteria (Stephenson, 1949), it is scarcely surprising that ammonia should figure so prominently in the nitrogen metabolism of the rumen. Shazly & Syngo (1950) have demonstrated the marked capacity of ruminal bacteria to deaminate amino-acids; when suspensions of the washed bacteria were incubated with acid-hydrolysed casein, up to 35 % of amino-acid N appeared as ammonia. In addition, the rumen bacteria possess desamidase which

actively produces ammonia from asparagine and glutamine (author's unpublished experiments). It is quite probable that some others of the N.P.N. substances occurring naturally in fodders may be degraded with the formation of ammonia, and to the degree that this occurs, these substances would contribute to the turnover of nitrogen in the rumen in exactly the same way as at least part of the amino-acids and amides. The available data are too inadequate to evaluate these processes.

It has now been established that the ruminant can survive on a diet in which virtually all the nitrogen is supplied in the form of urea (Loosli, Williams, Thomas, Ferris & Maynard, 1949), which is converted in the rumen into ammonia, but it is evident that a normal population of micro-organisms is not maintained in the rumen on such a diet (Gall, Thomas, Loosli & Huhtanen, 1951). It seems likely that many of the ruminal species are exacting in their requirements for amino-acids and that a diet which supplies little or no nitrogen in the form of protein or amino-acids, would lead to the disappearance of the exacting species and the survival or establishment of species which can use ammonia as the sole source of nitrogen.

The formation of ammonia in the rumen leads to two opposing nutritional tendencies. First, since substances such as urea, which are nutritionally valueless to the host, can be converted to ammonia and utilized for growth of bacteria, that is for synthesis of protein, which can be subsequently digested and used by the host, a gain of nitrogen accrues to the host animal. By contrast, the degradation of protein to ammonia, which can be directly absorbed from the rumen, implies a source of loss of nitrogen to the host animal. The interaction of these opposing tendencies is probably a major factor leading to the relative constancy of the biological value of food nitrogen (crude protein) for ruminants (Johnson, Hamilton, Mitchell & Robinson, 1942).

A provisional outline of the main events concerned in the digestion of protein in the rumen may be given as follows. Under ordinary conditions of feeding, the nitrogen entering the rumen will comprise chiefly protein together with varying amounts of non-protein nitrogenous substances as peptides, amino acids, amides, purines, pyrolles, simple bases such as choline and the betaines, inorganic N as ammonia, nitrates and nitrites, and traces of other substances. The nitrogenous bases and amino compounds may be deaminated while nitrates and nitrites are reduced to ammonia (Lewis, 1951). Ammonia is also produced by the degradation of proteins. In addition, small but significant amounts of nitrogen are added to the rumen contents by the saliva, in which the most important component is urea, which is readily converted into ammonia. Ammonia is utilized by the

micro-organisms for growth, together with amino-acids produced by the activity of the bacterial proteases. Protein leaving the rumen by passage in the ingesta to the more distal parts of the gastrointestinal tract consists of a mixture of undigested food protein and the protein of the micro-organisms. The ratio of these two forms of protein in the ingesta leaving the rumen has not yet been determined under any natural feeding conditions, but McDonald (1948*b*) has reported the extent of conversion of zein to microbial protein in sheep fed a partially purified diet. Ammonia is absorbed from the rumen and in part may return to the rumen, after passage through the liver, by secretion as urea in the saliva, while in part it would be excreted in the urine as urea. Some of the nitrogen utilized by the ruminal micro-organisms for growth would appear as nucleic acids (and other non-protein substances) which are probably of very limited, if any, value to the host animal. The biological value of protein or of any

other nitrogenous substance in the diet of a ruminant will be determined, in part, by the degree to which it is attacked and utilized by the ruminal micro-organisms.

SUMMARY

1. In the rumen fluid, ammonia constitutes the main component of the non-protein nitrogen, when the animal is fed natural diets or a diet in which casein is the main source of nitrogen. The insoluble protein, zein, is only slowly digested in the rumen.

2. Indirect evidence suggests that ammonia represents an important intermediate in the digestion of dietary protein and its utilization by the symbiotic micro-organisms of the rumen for their growth.

3. The implications of these observations on the role of micro-organisms in the digestion of protein in the rumen are discussed.

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Effect of Haemoglobin and other Nitrogenous Compounds on the Respiration of the Rhizobia

BY R. H. BURRIS AND P. W. WILSON

Departments of Biochemistry and Bacteriology, University of Wisconsin, Madison, U.S.A.

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Kubo (1939) established that the red pigment in leguminous root nodules is a haemoprotein with absorption characteristics almost identical with animal haemoglobin, and that the addition of the pigment to a suspension of soybean organisms enhanced their oxygen uptake on succinate. In a paper published in 1943 by Kasugai, Kubo & Tsujimura, but only recently available, these initial obser-

vations were extended to include pure cultures of the root nodule and other bacteria. The stimulation by haemoglobin was blocked by hydroxylamine, which combines only with ferric iron; this inhibition is surprising if the haemoprotein were merely oxygenated and deoxygenated, for the iron would then remain in the ferrous state. Despite this observed effect of hydroxylamine, Kasugai *et al.* concluded

that the function of the haemoprotein in the nodule is to store and transport molecular oxygen, a view independently proposed by Little & Burris (1947), who had extended the observations to stimulation of the respiration of a variety of organisms—*Rhizobium trifolii*, *Escherichia coli* and *Torula canadensis*. The stimulation was especially marked at a P_{O_2} of 0.01 atm. and was eliminated if the haemoprotein was denatured by heating.

Smith (1949), in a careful reinvestigation of the problem, noted that the respiration of intact nodules on the plant and excised whole and sliced root nodules was not inhibited by carbon monoxide, and on the basis of this and other evidence he concluded: 'The effect of haemoglobin in this and probably in the other experiments, including those at the low partial pressures of oxygen, is not connected with its ability to undergo oxygenation. A probable explanation is that *R. trifolii* and many other bacteria are able to break down haemoglobin and use it as a nitrogen source, so that in the presence of haemoglobin, the bacteria change over from a rate of oxygen uptake corresponding to a resting metabolism to a higher rate characteristic of a proliferating metabolism.'

Data gathered by us some years ago (Burris, 1940) and mentioned briefly in the review by Burris & Wilson (1939), together with some recent experiments with haemoprotein on the respiration of the rhizobia, are presented here in an examination of this conclusion of Smith.

EXPERIMENTAL METHODS

Effect of amino-acids. Various species of *R. trifolii* were grown in litre Roux flasks at 28° on a medium containing 10 g. Difco yeast extract (or 100 ml. of a hot water extract of fresh yeast), 0.5 g. K_2HPO_4 , 0.1 g. NaCl, 0.2 g. $MgSO_4 \cdot 7H_2O$, 0.5 g. $CaCO_3$, and 20 g. agar, per litre. Iron was supplied at 3 μ g./ml.; the medium was adjusted to pH 7.0. The fast-growing cultures were incubated for 2 days, the slow-growing cultures (soybean and cowpea organism) for 4 days. The cells were removed with a glass rod from each bottle of culture after about 10 ml. of the salt solution of Allison & Hoover (1934) had been added; then lumps of agar were removed from the suspension by filtration through cheesecloth. The cells were centrifuged, twice resuspended and recentrifuged, and finally stored at 3° suspended in the solution of Allison & Hoover.

Measurements were made in Warburg respirometers at 34°. Unless otherwise stated, the Warburg flasks contained 1 ml. of 0.066M-phosphate buffer, pH 6.5, 1 ml. of bacterial suspension, 0.5 ml. of 0.04M-substrate and 0.5 ml. of inhibitor, stimulator or water; 0.15 ml. of 20% KOH was placed in the central alkali well.

Effect of haemoglobin. Haemoglobin prepared from hog blood by the method of Welker & Williamson (1920) was dialysed before use. Heat-denatured haemoglobin was used in control flasks; a haemoglobin solution was denatured by placing it in a boiling-water bath for 1 min., homogenizing

with a Potter-Elvehjem homogenizer (Umbreit, Burris & Stauffer, 1949, p. 136), heating again for 1 min. and again homogenizing. Tests with haemoglobin were made at 30° in respirometer vessels of 6–8 ml. capacity. The main chamber of the vessel contained 0.5 ml. 3% glucose and 0.5 ml. haemoglobin, denatured haemoglobin or water; 0.5 ml. of cell suspension in 0.1M-phosphate buffer of pH 7.3 was placed in the side sac, and 0.05 ml. 20% KOH was placed in the centre well. These solutions corresponded to those used by Smith (1949). Gas mixtures were made from air and tank gases. When mixtures with a P_{O_2} of 0.01 atm. were desired, water was displaced from two water-filled gas bottles with high purity N_2 ; 5% of the atmosphere of one bottle was then replaced with tank CO. The two bottles were then connected with a T-tube and 0.05 atm. was removed simultaneously from both; when this was replaced with air the gas mixtures in the bottles had a P_{O_2} of 0.01 atm. Such a procedure assured that the same percentage O_2 would be present in both gas mixtures. Analysis of two such mixtures in the respirometers indicated that the mixture with CO had 1.32% O_2 and the mixture without CO had 1.30% O_2 . Respirometer vessels were gassed from these mixtures as described by Umbreit *et al.* (1949, p. 44).

RESULTS

Effect of amino-acids

Table 1 presents data on the effect of nitrogenous compounds on the respiration of root-nodule bacteria representing four cross-inoculation groups. It is evident from the table that *Rhizobium meliloti* 100, *R. trifolii* 205 and 209 and *R. leguminosarum* 317 respond in a reasonably similar manner to the compounds tested, although strains 100 and 317 respond less vigorously than 205 and 209. Compounds which are especially stimulatory include the amino-acids glycine, alanine, aspartic acid, glutamic acid and arginine, the dipeptide glycylglycine, and the amide asparagine. The mixture of amino-acids from casein hydrolysate is distinctly more stimulatory than single amino-acids. Intermediate and rather uniform responses were obtained with the alpha amino-butyric, -valeric and -caproic acids. Cysteine, glycine anhydride, calcium nitrate and sodium nitrate stimulated slightly or were inhibitory. Except for strain 602 the response with ammonium nitrate, the best inorganic stimulator tested, was comparable to that obtained with the more active amino-acids. The apparent, more striking, stimulation of the cowpea organism on amino-acids can be attributed to the fact that glucose is a poor substrate for this culture; hence any direct oxidation of the added amino-acid appears as a great stimulation.

Initially (first hour) the stimulation was independent of the concentration of the added nitrogenous compound. With ammonium nitrate, for example, the following stimulation was noted: 0.02M, 45%; 0.01M, 48%; 0.001M, 40%. Similar data were obtained with aspartic acid. The total stimulation (total oxygen uptake in 3.5 hr.)

Table 1. *Effect of nitrogenous compounds on the respiration of the rhizobia supplied with glucose*

(All figures as percentage stimulation over respiration on 0.0066M-glucose alone. Negative values indicate inhibition. Nitrogenous compounds in 0.001M concentration. q_{O_2} indicates oxygen uptake in μ l./mg. bacterial N/hr.)

Nitrogen compound	Strain of organism*				
	100	205	209	317	602
Glycine	21	35	43	15	24
Alanine	19	30	33	23	35
Aspartic acid	14	24	27	14	111
Asparagine	22	43	32	22	232
Glutamic acid	15	40	39	13	137
Leucine	11	23	21	7	58
Aminobutyric acid	22	25	21	13	158
Aminovaleric acid	16	26	21	10	87
Aminocaproic acid	16	26	22	17	125
Aminocaprylic acid	5	23	8	9	146
Cysteine	-13	-9	-8	8	-6
Histidine	5	26	33	16	-5
Arginine	31	30	30	11	-6
Tryptophan	-2	8	12	0	29
Glycylglycine	25	29	26	27	41
Glycine anhydride	—	-1	2	1	8
1/1200 dry wt. casein hydrolysed	35	80	40	31	217
NH ₄ NO ₃	9	30	37	16	-8
Ca(NO ₃) ₂	-3	-6	-2	4	-5
(NH ₄) ₂ HPO ₄	-1	20	28	20	-12
(NH ₄) ₂ SO ₄	1	25	21	21	-6
NaNO ₂	-2	-1	7	3	-20
q_{O_2} on glucose	454	319	461	442	192

* *R. meliloti* 100; *R. trifolii* 205 and 209; *R. leguminosarum* 317; *Rhizobium* sp. (cowpea) 602.

Table 2. *Oxidation of glucose by Rhizobium trifolii* 205 in the presence and absence of glycine

Substrate	Glucose used		Uptake of oxygen		
	mg.	O ₂ equiv. (μ l.)	μ l.	From glucose (μ l.)	Oxidation of glucose (%)
		(μ l.)			
		First hour			
Glucose	1.089	813	436	436	54*
Glucose plus glycine	0.675	504	553	477†	95
		Second hour			
Glucose	1.617	1208	192	192	16
Glucose plus glycine	0.948	708	477	385	54

0.0066M-Glucose and 0.01M-glycine

* (O_2 from glucose \times 100)/(O_2 equiv. of glucose disappearing). No corrections for endogenous respiration.

† Calculated from observed minus that equivalent to glycine oxidized based on NH₃-N liberated: first hour, 76 μ l. O_2 ; second hour, 92 μ l. O_2 .

likewise was relatively independent of the concentration within rather wide limits. With glycine the total stimulation was approximately 400 μ l. O_2 between concentrations of 0.004 and 0.02M. Below and above this range the total stimulation decreased rapidly, although the initial stimulation of about 40% was observed in concentrations ranging from 0.00033 to 0.5M. Stimulation of young cultures (24 hr.) was usually somewhat greater than that of older, but this may reflect only that the overall metabolism is greater in young cells.

Burris & Wilson (1939) reported that stimulation is evident almost immediately upon the addition of amino-acids. Since this point is of some importance

in the argument that the observed response cannot be attributed to proliferation of the cells, the experiment was repeated. A heavy suspension of *R. trifolii* was used so that accurate readings could be obtained at 4 min. intervals. After a preliminary period to determine the rate of oxygen uptake on glucose alone, the nitrogenous compounds were added from the side arms. A slight lag was observed in the response at the 4 min. reading, but by 8 min. the final rates had been attained.

Mechanism of action. To gain an insight into possible mechanisms of stimulation by amino-acids, analyses were made at intervals in an experiment with *R. trifolii* 205. It appears that the following

Table 3. Oxidation of glucose and glycine alone and together by *Rhizobium trifolii* 205

(All values for initial 120 min. of experiment and based on content of single Warburg flask containing 1 ml. 0.066M-phosphate buffer, pH 6.5, 0.5 ml. 0.06M-glycine solution (or water), 0.5 ml. 0.04M-glucose solution (or water), 1 ml. suspension in side arm. Theoretical oxygen equivalent of glucose added: 2688 μ l. Oxygen equivalent of amino-acid disappearing based on complete oxidation of carbon in glycine.)

Substrate	O ₂ uptake (μ l.)	α -Amino N disappearing		NH ₃ -N appearing	
		μ mol.	O ₂ equiv. (μ l.)	μ mol.	O ₂ equiv. (μ l.)
Glucose	538	—	—	—	—
Glycine	272	8.58	288	10.0	336
Glucose plus glycine	819	7.95	266	5.6	188

reactions may be important: (a) sparing of glucose by oxidation of glycine; (b) oxidation of glycine with liberation of ammonia; (c) stimulation of oxidation of both glucose and endogenous substrates by the ammonia. It is difficult to assign a quantitative value to each of these, but an estimate of their importance can be obtained from the data in Table 2. During the first hour the stimulation of uptake of oxygen by the presence of glycine was 117 μ l.; an uptake of only 76 μ l. would be needed for complete oxidation of the glycine calculated from the ammonia liberated. The corresponding figures for the second hour were 285 and 92 μ l.

These data, suggesting an increased oxygen uptake from glucose, could result from a sparing action of the glycine on the glucose in the sense that the oxidation of the amino-acid decreases the quantity of glucose that is oxidatively assimilated. A quantitative estimate of this effect is given by the percentage of glucose that disappears accounted for by the oxygen used (last column of Table 2). That the oxidation of endogenous substrates as well as the glucose may be stimulated by the simultaneous oxidation of glycine is indicated by the following observation: during this trial in 150 min. the oxidation of glycine required 706 μ l. of oxygen, whereas the endogenous respiration was 202 μ l. The difference, 504 μ l., is considerably higher than the value of 445 μ l. calculated from the 13.2 μ mol. of ammonia liberated from the glycine.

The foregoing interpretation is not unequivocal, however, since the quantity of glycine that disappears has been calculated from the ammonia appearing, clearly a minimum value, as part of the liberated ammonia may have been subsequently assimilated. Another experiment was made, therefore, in which both ammonia appearing and amino nitrogen disappearing were followed. As in the first experiment ammonia N was liberated less rapidly in the presence of glucose, but the rate of disappearance of α -amino N was not affected (Fig. 1). Moreover, the data on uptake of oxygen summarized in Table 3 are consistent with the view that the observed stimulation in respiration arises from the simultaneous and independent oxidation of glycine and

glucose. This simple interpretation, however, disregards the role of the endogenous respiration and the marked difference in the ammonia found in the presence and absence of glucose. The endogenous respiration was 106 μ l. O₂ in 120 min. The ammonia found when glycine alone was the substrate was

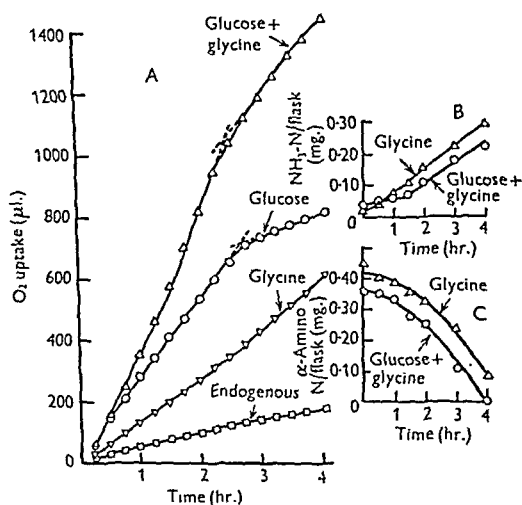


Fig. 1. A, effect of glycine on the oxidation of glucose. B, release of ammonia N from glycine in the presence and absence of glucose. C, disappearance of α -amino N in the presence and absence of glucose.

1.42 μ mol. greater than the amino N that disappeared, whereas in the presence of glucose it was 1.35 μ mol. less. A reasonable explanation appears to be that the excess ammonia arises from endogenous respiration and the deficiency from assimilation of liberated ammonia—an assimilation that is accompanied by a stimulation in oxygen uptake. The slight increase in the rate of oxygen uptake evident in the glucose plus glycine curve during the first 2 hr. (Fig. 1A) supports this view. This slow increase in rate attributable to growth during the 2 hr. period, however, is not to be confused with the quantitatively much greater stimulation which appears almost immediately on the addition of various nitrogenous compounds.

Effect of haemoglobin

The data in Fig. 2 summarize the results of an experiment designed to repeat the tests of Smith (1949) in which the significant observation was made that haemoglobin even in the presence of excess carbon monoxide stimulated respiration of the root-nodule bacteria. Each point on the graph represents an average reading from two flasks. From the slopes

of the lines the values for rates of oxygen uptake given in the legend of the figure are obtained. Several conclusions can be drawn from these results which agree with the parallel data of Smith; however, our experiment includes additional tests at a low P_{O_2} . First, the percentage stimulation of oxygen uptake is somewhat greater at the lower P_{O_2} . With horse haemoglobin, Smith noted that the stimulation of oxygen uptake was 111% at a P_{O_2}

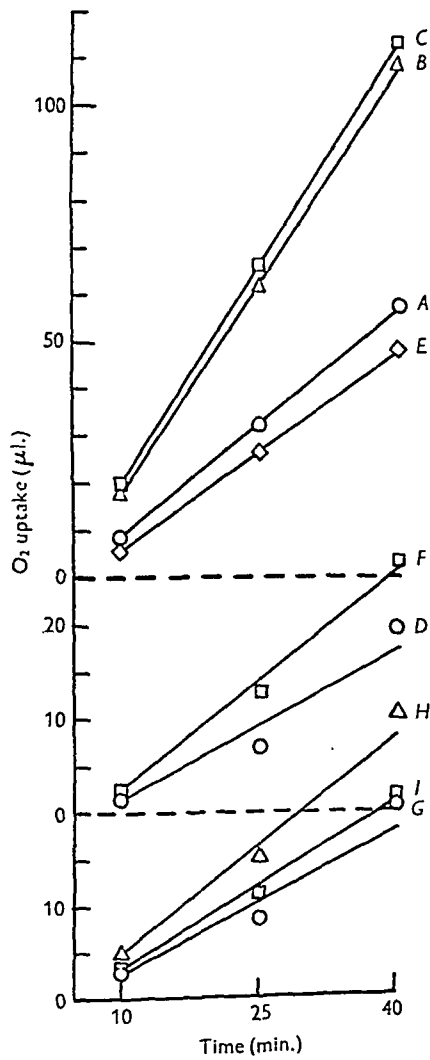


Fig. 2. Effect of hog haemoglobin on the oxygen uptake of *Rhizobium leguminosarum*.

Curve	Added solution	Atmosphere	O ₂ uptake (μl./hr.)	Rate of O ₂ uptake relative to appropriate control
		Air	99	100
A	Water	Air	182	184
B	Haemoglobin	Air	186	188
C	Haemoglobin	Air with P_{CO} 0.05	32	100
D	Water	P_{O_2} 0.01	85	266
E	Haemoglobin	P_{O_2} 0.01	47	147
F	Denatured haemoglobin	P_{O_2} 0.01, P_{CO} 0.05	31	97
G	Water	P_{O_2} 0.01, P_{CO} 0.05	47	147
H	Haemoglobin	P_{O_2} 0.01, P_{CO} 0.05	36	113
I	Denatured haemoglobin			

of 0.01 atm. as compared with 35% at a P_{O_2} of 0.20 atm. Second, a P_{CO} of 0.05 atm. in air (P_{O_2} , 0.20 atm.) does not reduce the stimulation by haemoglobin, but in an atmosphere with a P_{O_2} of 0.01 atm., carbon monoxide does reduce the observed stimulation. Third, in contrast with the results found by Smith (1949) the heat-denatured haemoglobin we have used in these experiments did not inhibit oxygen uptake. This point is important, since if such inhibition occurs it could explain the results of Little & Burris (1947) as arising from a faulty control. In the particular experiment shown in Fig. 2 the denatured haemoglobin was, if anything, stimulatory (although much less than the undenatured haemoglobin), but in other trials denatured haemoglobin exerted neither definite stimulation nor inhibition of respiration in either air or at a P_{O_2} of 0.01 atm. Finally, in agreement with the findings of Smith, we have noted that carbon monoxide *per se* at a P_{CO} of 0.05 atm. has little inhibitory effect on the rate of oxygen uptake in the absence of haemoglobin.

A single test was made on the influence of the P_{O_2} on the stimulation of respiration by amino-acids, but no consistent correlation was observed.

DISCUSSION

In seeking to interpret the results of the various investigations of the role of haemoglobin in nitrogen fixation by leguminous plants, fortunately, few serious disagreements exist among the experimental findings. Haemoglobin definitely stimulates respiration of the root-nodule bacteria, but this stimulation is non-specific as demonstrated by tests with other bacteria. It appears unlikely that the observed stimulation can be associated with an oxygenation process in view of the results with carbon monoxide noted by Smith and confirmed in this paper. The inhibition of the stimulation by hydroxylamine reported by Kasugai *et al.* might also be included as evidence for rejection of the explanation based on oxygenation.

If haemoglobin is not performing its usual role, the proposal of Smith that the stimulation arises from an effect of nitrogenous compounds in changing the metabolism from that of resting cells to that of proliferating cells deserves serious consideration. In recent years, several reports have appeared demonstrating that the respiration of various bacteria is increased in the presence of nitrogenous compounds, including ammonia. Armstrong & Fisher (1947) observed that when young cells of *Esch. coli* were supplied with ammonium chloride and a source of carbon, their rate of oxygen consumption increased until the ammonium ion was exhausted, then the rate fell to 45% of the maximum. McLean & Fisher (1947, 1949) found that

an increased rate of respiration was proportional to the conversion of ammonia to cellular nitrogen in *Serratia marcescens* and concluded that 'the bacterial protoplasm consumed oxygen at two rates, depending upon whether it was actively growing or was in a resting condition'.

In the response of the rhizobia to the added sources of nitrogen, 'growth' in the usual sense can be ruled out, since: (a) the response is immediate, whereas the usual generation time of the organism is 2-3 hr.; (b) the quantity of nitrogen added is small—about 25 μ g. in comparison with the 1200 μ g. present in the suspension; (c) the uptake is essentially linear with no indication of logarithmic growth. When observations are extended over a long period, some increase in the rate is observed (Fig. 1A), but this increase is small and distinct from the increase observed immediately after addition of the source of nitrogen. Apparently many washed bacterial cells, including the rhizobia, devoid of a source of nitrogen carry on a resting metabolism of non-nitrogenous substrates, and when a nitrogenous compound is supplied they immediately assume a more rapid metabolism. As has been demonstrated in this paper, with the rhizobia this stimulation may arise primarily, though not exclusively, from simultaneous oxidation of an added source of carbon when an amino-acid is used as the nitrogenous compound. Obviously, this is not the explanation when the compound is inorganic. The response other than simple oxidation can be ascribed to a 'growth process', however, only in a special sense, as it essentially does not increase with time. It may be preparatory for an increase in size and number which follows some time later—one cannot help noticing that the effects resemble the so-called specific dynamic action of proteins and amino-acids in the animal cell rather than true growth.

Although this effect of nitrogenous compounds in stimulating respiration is an attractive explanation of the results obtained with haemoglobin and the rhizobia, it leaves several experimental facts unexplained. First, it must be assumed that native haemoprotein will serve as a nitrogen source for the rhizobia, but heat-denatured haemoprotein will not. The fact that the proteolytic enzymes of the rhizobia are extremely weak (Berger, Johnson & Peterson, 1938) makes it improbable that they would use either form of the intact protein. Dialysed egg white was not active in stimulating respiration. Secondly, the curious fact remains that at a low P_{O_2} the effect of haemoglobin is enhanced (Little & Burris, 1947). In a single experiment reported by Smith (1949) the percentage stimulation in uptake of oxygen was over 3 times as great at a P_{O_2} of 0.01 atm. as at 0.20 atm. In limited trials we were unable to obtain a similar effect with a low P_{O_2} on stimulation of respiration by amino-acids. Finally, although

carbon monoxide does not inhibit the stimulation when the experiments are made at a P_{O_2} of about 0.20 atm., it does appear to inhibit when the P_{O_2} is 0.01 atm. This effect may be important in the soil, where the P_{O_2} is considerably lower than in air.

In summary, the observed stimulation of respiration by haemoglobin apparently does not involve its acting as a carrier of molecular oxygen, but neither does it appear to be an experimental artifact. Its action may require a change in valence of the iron, but whether this change is directly associated with nitrogen fixation or indirectly through respiration is as yet unresolved. In either case the mechanism remains obscure. Although the view that haemoglobin in the nodule participates directly in the fixation reaction has considerable indirect support, its plausibility would be materially increased if the possibility of an effect on respiration could be eliminated. At present the best outlook for this appears to be the resolution of the difficulties just discussed.

SUMMARY

1. Certain amino-acids and inorganic nitrogen salts increased the respiration of washed suspensions of root-nodule bacteria furnished with glucose. This stimulation was immediate and did not increase appreciably with time during the period of measurement (up to 4 hr.).

2. The initial increase in rate was constant over a wide range of concentrations, but the total increase of oxygen uptake was dependent upon the

concentration of nitrogenous compound supplied. Young cells were stimulated somewhat more than old cells.

3. In the presence of glycine, the uptake of oxygen by *Rhizobium trifolii* furnished with glucose was more rapid than in its absence; much, though not all, of this stimulation apparently arises from oxygen used in the oxidation of the glycine.

4. The respiration rate of washed cells of *R. leguminosarum* supplied with glucose was enhanced by the addition of hog haemoglobin. The percentage increase was greater at a P_{O_2} of 0.01 atm. than at 0.20 atm. A P_{CO} of 0.05 atm. did not reduce this stimulation at a P_{O_2} of 0.20 atm. but did so at a P_{O_2} of 0.01 atm. Heat-denatured haemoglobin had little influence on the rate of oxygen uptake.

5. Even though the respiration of the rhizobia is enhanced by added nitrogenous compounds, the assumption that haemoglobin increases respiration by the same mechanism does not necessarily follow. Certain experimental findings are inconsistent with this explanation.

6. Although direct participation of the nodular haemoglobin in the nitrogen-fixation reaction appears to be an attractive and plausible function, examination of the evidence to date suggests that other alternatives have not been eliminated.

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The Characterization and Assay of Enzymes in Rat Adrenal Cortex

1. ESTERASE AND PHOSPHATASE ACTIVITIES

By J. J. GORDON

*Biochemical and Endocrinological Research Department, Bristol Mental Hospitals,
Fishponds, Bristol*

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There is evidence that significant changes may take place in the activities of enzymes present in secreting glands under the influence of regulatory hormones. Gutman & Gutman (1938) reported increases in the acid phosphatase activity of prostate during the development of the epithelia induced by testosterone. According to Fuenzalida (1949), prolonged oestrogen treatment causes an increase in alkaline phosphatase activity in guinea pig uterus. Large increases in arginase and alkaline phosphatase activities in the mammary gland during lactation have been found by Folley & Greenbaum (1947).

Evidence regarding the nature of similar changes taking place in the adrenal cortex has been largely histological. Characteristic alterations in lipid metabolism in this gland in the rat, following hypophysectomy, were indicated by the work of Reiss, Balint, Oestreicher & Aronson (1936). Variations in alkaline phosphatase content under different endocrine conditions have been investigated in the mouse and rat by Elftman (1947) and by Dempsey, Greep & Deane (1949). Chemical studies, however, have so far been limited, though isolated observations, sometimes of a qualitative kind, have from time to time been reported. The presence of a lipase (i.e. tributyrinase) in the tissue was demonstrated by Cheboksarov & Malkin (1927) and by Scoz & Mariani (1939); this would seem to be of importance in relation to the lipid changes observed by Reiss *et al.* (1936). Chemical proof of the existence of an adrenal cortical phosphomonoesterase has also been furnished (Granger & Bessoles, 1945).

A knowledge of the properties of the various enzyme systems present in the adrenal cortex, and the manner in which they are influenced by different endocrine factors, may be important in connexion with the processes governing the secretory activity of the gland. Investigations on these lines have accordingly been initiated, and the present paper contains an account of the experimental work so far carried out to characterize the lipase and phosphatase activities shown by aqueous homogenates of adrenal cortical tissue, and to devise suitable methods for their accurate assay in small quantities of tissue. Since tributyrin has been used as the principal test substrate for lipase, the latter will be

referred to throughout as the 'aliphatic esterase'. Some related observations on cholinesterase activity are included.

METHODS

Preparation of tissue homogenates

The demedullated adrenals of albino rats have been used exclusively as a source of enzyme. The rats were killed by decapitation, the carcasses exsanguinated, and the adrenals rapidly removed, cleaned from adherent fat, then opened laterally with fine scissors, and the medullae removed from both halves. The pooled demedullated adrenal tissue obtained from a number of rats was weighed, rapidly washed with cold water by decantation to remove excess blood, and homogenized in cold distilled water in a Potter-Elvehjem (1936) homogenizer. After making to the appropriate volume with distilled water and mixing thoroughly, the homogenate was allowed to stand 1-2 min. to allow the capsular debris to settle, then decanted carefully off.

Aliphatic esterase and cholinesterase activity

These were usually followed at 37° by use of the Ammon (1933) technique. Tissue homogenate (1 ml.) containing a suitable wet weight of tissue (12-18 mg. for aliphatic esterase and 40 mg. for cholinesterase) was incubated in NaHCO_3 (0.027M) medium with the ester, the total fluid volume being 3 ml., in an atmosphere of 5% CO_2/N_2 . CO_2 output was measured at regular intervals for a period of 30-60 min. and corrections made in each case for spontaneous hydrolysis. Activities are expressed as $\mu\text{l. CO}_2/30 \text{ min./g.}$ In preliminary experiments, aliphatic esterase activity was tested against a series of simple esters; most of the experiments with this system, however, were performed with tributyrin as substrate. 0.2 ml. of the pure ester (more than sufficient to saturate the system) was used. For cholinesterase activity, the substrate consisted of acetylcholine (ACh, 6-12 mM), acetyl- β -methylcholine (MeCh, 12 mM) or benzoylcholine (BCh, 6 mM). It was confirmed that the ester concentrations were in every case adequate to ensure maximal hydrolysis rates. In some experiments selected inhibitors were included in the incubation mixture.

For following the hydrolysis of the glyceryl acetates, a variation of the titration method of Knaffl-Lenz (1923) was employed at room temperature, on account of the rather high rates of spontaneous hydrolysis shown by these esters in NaHCO_3 at 37°. The homogenate (1 ml. of 40 mg./ml.) was diluted with water (5 ml.) and 0.3 ml. bromothymol blue (0.1%) added. The ester (1 ml.) was added, the mixture shaken, and the pH adjusted to neutral by cautious addition of NaOH solution (0.01N). Enzyme and substrate blanks

were also set up. The time was noted, and the pH of the mixtures continuously adjusted by additions of 0.01N-NaOH from a micro-burette. The total volume of alkali was recorded at intervals. Each of the reaction mixtures in a run was matched against a colour standard consisting of 6 ml. 0.033M-phosphate pH 7.4 buffer + 1 ml. homogenate + 0.3 ml. bromothymol blue.

Phosphatase activity

Four types of phosphatase activity were studied—alkaline and acid phosphomonoesterase, inorganic pyrophosphatase and adenosinetriphosphatase (ATP-ase). Activities were measured at 37° by estimation of orthophosphate liberation, using as substrates sodium glycerophosphate, $\text{Na}_2\text{P}_2\text{O}_7$, or ATP. In the standard procedure the tissue suspension was added to 2 ml. Walpole acetate or Michaelis veronal buffer solution. (The pH values recorded were those of the final mixtures, including substrate and enzyme.) The total volume of the reaction mixture in each case, including activating substances where necessary, was 4 ml. Appropriate enzyme and substrate blanks were also set up. The conditions employed for the different types of phosphatase activity were as follows:

Alkaline phosphomonoesterase (pH 9.6). Tissue concentration was 4–7 mg./ml., substrate concentration 6–20 mM, and incubation period 1 hr. MgCl_2 (5 mM) was included as an activator.

Acid phosphomonoesterase (pH 5.2). Tissue concentration was 4–7 mg./ml., substrate concentration 6–18 mM, and incubation period 2 hr. MgCl_2 was omitted.

Pyrophosphatase (pH 7.4). Tissue concentration was 1–1.3 mg./ml., substrate concentration 1 mM, and incubation period 20–30 min. MgCl_2 (8 mM) and cysteine (CySH , 10 mM) were included as activators.

Adenosinetriphosphatase (pH 7.4). Tissue concentration was 1.5–2.0 mg./ml., substrate concentration 1.7–3.0 mM, and incubation period 15 min. MgCl_2 (4 mM) and CySH (10 mM) were included as activators.

Variations in the above conditions were made to establish optimal conditions for enzyme assay. In experiments where inhibitors were used, they were included in the incubation mixture prior to addition of substrate, except in the case of alcohol, where the conditions employed by Abul-Fadl & King (1949) were employed.

The tubes containing the reaction mixtures were shaken at 37° for the appropriate incubation period and then treated with 10% CCl_3COOH (2 ml.) and allowed to stand 20 min. before filtration. Orthophosphate was estimated in suitable portions (usually 3 ml.) of the filtrate by the method of Fiske & Subbarow (1925), the colours being read on the Spekker photoelectric absorptiometer, using Ilford orange filters. Results are expressed as μmol . substrate decomposed/30 min./g. wet wt. tissue. (In the case of ATP-ase, it was assumed that only one orthophosphate group was liberated during the short period of incubation employed.) It was confirmed that none of the substances used as inhibitors was able to interfere with the Fiske-Subbarow estimation under the conditions used.

Adenosinetriphosphatase. A commercial sample of the barium salt was washed with ice-cold pH 4.0 acetate buffer (0.1M) as recommended by Kornberg (1950) and converted to the sodium salt by the method of Bailey (1942). The concentration was checked by a 7 min. hydrolysis test.

Glycerophosphate. The sample used was found to contain 8.7% of the α - and 91.3% of the β -isomer, when analysed by the method described by Motzok & Wynne (1950).

RESULTS

Hydrolysis of aliphatic and choline esters

Initial rates of hydrolysis shown by a series of simple esters of primary alcohols, compared with tributyrin, are shown in Fig. 1. Only *n*-butyl *n*-propionate showed a hydrolysis rate higher than tributyrin, while the methyl, ethyl, *n*-propyl and *n*-butyl esters of *n*-butyric acid were all split at considerably lower rates. Rates of hydrolysis of

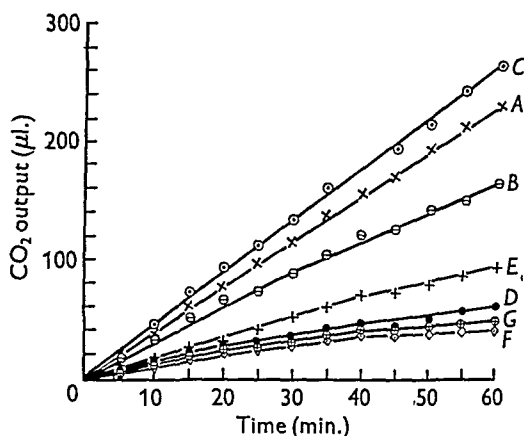


Fig. 1. Hydrolysis of aliphatic esters by rat adrenal cortex at 37° (measured manometrically). Tissue homogenate with 0.066% (v/v) ester. A, tributyrin; B, *n*-butyl acetate; C, *n*-butyl propionate; D, *n*-butyl butyrate; E, *n*-propyl butyrate; F, ethyl butyrate; G, methyl butyrate.

glyceryl acetates compared with tributyrin, measured by the titration technique, are illustrated in Fig. 2. The hydrolysis rates of monoacetin and diacetin were much lower than for triacetin, which itself showed a rate of hydrolysis roughly comparable with, though rather less than, that of tributyrin. In view of the relatively greater activity towards the triglycerides, the possibility of the enzyme system acting on natural fats such as triolein was examined. There was, however, no evidence of any measurable activity towards emulsified triolein, when tested by the methods described, or when the method of Willstätter, Waldschmidt-Leitz & Memmen (1923) was applied. It is evident that the lipolytic activity of the adrenal cortex is of a different character from that of the pancreas; it appears, in fact, to bear a closer resemblance to the plasma esterase studied by Adams & Whittaker (1949). The activity was proportional to tissue concentration over the range 1–7 mg./ml., so that accurate assays of activity levels are possible.

Compared with the hydrolysis of tributyrin the rates of hydrolysis of choline esters are low. A typical experiment gave activities of 8950 with tributyrin, 635 with ACh, 338 with MeCh, and 150 with BCh. The higher activity with MeCh compared

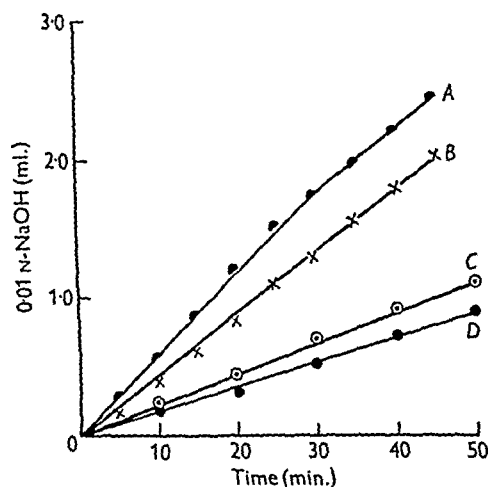


Fig. 2. Hydrolysis of glycerides by rat adrenal cortex at 20° (measured titrimetrically). Tissue homogenate with 0.14% (v/v) ester. A, tributyrin; B, triacetin; C, diacetin; D, monoacetin.

with BCh has been previously noted for adrenal cortex by Sawyer & Everett (1947) and for whole adrenals by Ord & Thompson (1950). This observation would appear to place it in the 'specific' cholinesterase class, according to the nomenclature of Mendel & Rudney (1943).

Effects of inhibitors. To assist in the further

characterization of these esterase systems the influence of a number of substances on the activity was studied. Substances tested were the sodium salts of bile acids, which were found to activate pancreatic lipase (Holwerda, 1938); also benzaldehyde, sodium fluoride and copper sulphate, all of which inhibit pancreatic lipase (Weinstein & Wynne, 1935). In addition, it was of interest to test the effects of certain cholinesterase inhibitors, in view of the relatively greater activity of the enzyme towards MeCh, compared with BCh. Substances of this type studied were eserine, which inhibits both specific and non-specific cholinesterases (Hawkins & Mendel, 1946); the compound Nu 1250, i.e. the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide, which is regarded as a selective inhibitor of specific cholinesterase (Hawkins & Mendel, 1949); and two compounds which show selective toxicity to the non-specific cholinesterase, i.e. the compounds Nu 683 (the dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide, Hawkins & Gunter, 1946) and 2987 R.P. (*N*-diethylaminoethylphenothiazine, Gordon, 1948). ACh only was used as substrate for cholinesterase in these experiments.

The results are seen in Table 1, and reveal several interesting features. The hydrolysis of tributyrin was strongly inhibited by the sodium salts of cholic, deoxycholic and tauroglycocholic acids, but only slightly affected by benzaldehyde—this contrasts with the behaviour of the lipase of pancreas, though the adrenal cortex enzyme is inhibited by sodium fluoride and by copper sulphate. None of the cholinesterase inhibitors produced any marked effect on the hydrolysis of tributyrin. Cholinesterase

Table 1. *Effects of various substances on aliphatic esterase and cholinesterase activities of rat adrenal cortex at 37°*

Added substance	Concn. (mm)*	Inhibition of activity (%)	
		Aliphatic esterase (0.066% tributyrin as substrate)	Cholinesterase (12 mm-ACh as substrate)
Sodium cholate	7	—	14
Sodium deoxycholate	2.4	58	2
Sodium tauroglycocholate	3	91	0
Benzaldehyde	—	89	0
CuSO ₄	0.17% (v/v)	12	18
NaF	0.1	94	10
2987 R.P.	40	50	54
2987 R.P.	0.022	—	52
2987 R.P.	0.1	—	72
Nu 1250	0.3	0	—
Nu 683	5 × 10 ⁻³	4	24†
Nu 1250	3 × 10 ⁻³	5	93†
Nu 683	5 × 10 ⁻⁵	—	2
Eserine sulphate	5 × 10 ⁻⁵	—	48
	0.1	9	72

* Except where stated otherwise.

† Using 6 mm-ACh.

activity, on the other hand, was uninfluenced by the substances which inhibited tributyrin hydrolysis, with the exception of sodium fluoride. The effects of the cholinesterase inhibitors on cholinesterase activity were somewhat unexpected. 2987 R.P. inhibits strongly in a concentration of 0.022 mM, though concentrations up to 0.5 mM were previously found ineffective against specific cholinesterase in brain or plasma (Gordon, 1948). Only moderate inhibition was caused by 0.005 mM-Nu 1250, which in 0.001 mM concentration almost completely inhibits specific cholinesterase in brain and erythrocytes (Hawkins & Mendel, 1949). Nu 683, however, in similar concentrations, produced marked inhibition. These findings make it difficult to classify adrenal cortical cholinesterase on the basis of Mendel & Rudney's (1943) system. At the same time, it is clear that the choline esterase and aliphatic esterase systems are distinct from each other.

Phosphatase activities

Effect of pH on activity. Phosphomonoesterase activities at various pH values, with glycerophosphate as substrate, are indicated in Fig. 3. This type

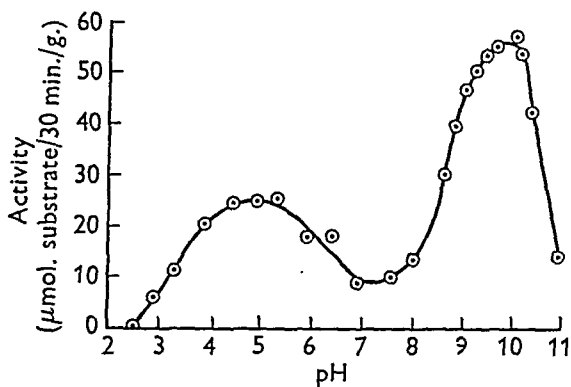


Fig. 3. Effect of pH on phosphomonoesterase activity of rat adrenal cortex at 37°. Tissue homogenate with 6 mM-sodium glycerophosphate. Mg^{++} absent.

of activity shows the usual characteristic pH optima in the acid and alkaline regions, as in most animal tissues. Only one optimum pH value was found in the acid region; there was no evidence of a second acid optimum, as has been found for erythrocytes (Roche, Thoai & Baudouin, 1942; Abul-Fadl & King, 1949). With sodium pyrophosphate or ATP as substrates, the optimum pH was 7.4 (Fig. 4). The presence of a strong ATP-ase activity in adrenals has already been reported (Zeller, 1948).

Influence of substrate concentration. The effects of substrate concentration are seen in Figs. 5 and 6. Well defined optimal substrate concentrations were obtained with pyrophosphatase (0.3 mM) and ATP-ase (1.5 mM). The effects with acid and alkaline phosphomonoesterases were not so clear-cut; on

the basis of these results, however, a substrate concentration of 18–20 mM provides near-optimal conditions.

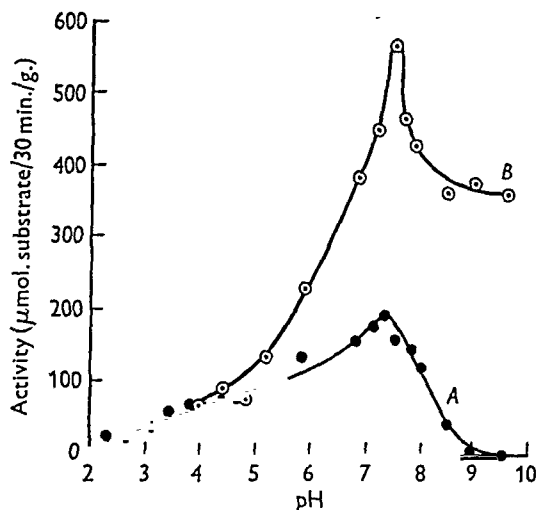


Fig. 4. Effect of pH on phosphatase activity of rat adrenal cortex at 37°. A, 1 mM- $Na_4P_2O_7$ + 8 mM- Mg^{++} ; B, 1.7 mM-ATP + 4 mM- Mg^{++} .

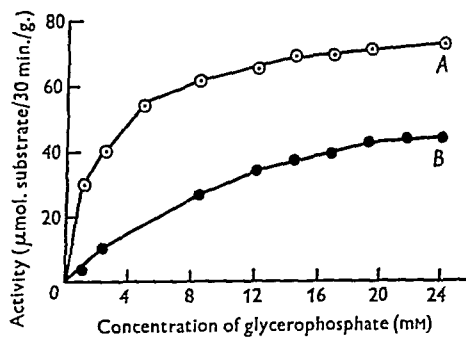


Fig. 5. Effect of substrate concentration on phosphomonoesterase activity of rat adrenal cortex at 37°. Sodium glycerophosphate as substrate. A, pH 9.6; B, pH 5.2.

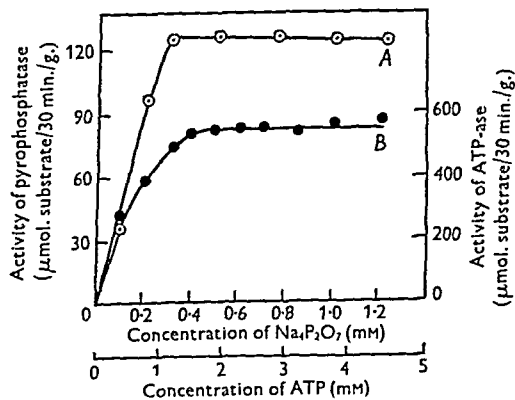


Fig. 6. Effect of substrate concentration on phosphatase activity of rat adrenal cortex at 37° and pH 7.4. A, $Na_4P_2O_7$ with 20 mM- Mg^{++} . B, ATP with 4 mM- Mg^{++} .

Activation by Mg^{++} . The behaviour of the different systems towards magnesium is similar to that found in other tissues—alkaline phosphatase is activated appreciably by Mg^{++} in concentrations of 1–10 mM, but acid phosphatase is not perceptibly influenced. Mg^{++} is essential for the activity of pyrophosphatase and shows an optimal effect at 4 mM. ATP-ase is markedly stimulated by Mg^{++} , the effect being maximal at 3–4 mM, but declining again at higher concentrations. Ca^{++} also activates ATP-ase, but not to the same extent as Mg^{++} .

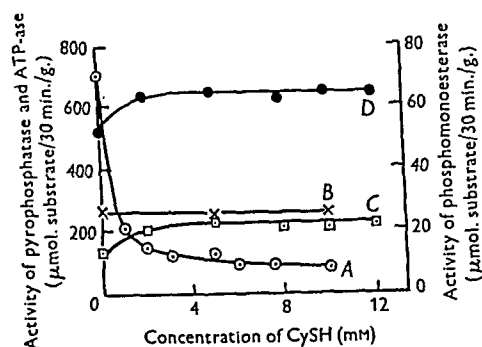


Fig. 7. Effects of cysteine on phosphatase activities of rat adrenal cortex at 37°. A, 12 mM-sodium glycerophosphate + 5 mM- Mg^{++} , pH 9.6; B, 18 mM-sodium glycerophosphate, pH 5.2; C, 1 mM- $Na_4P_2O_7$ + 8 mM- Mg^{++} , pH 7.4; D, 1.9 mM-ATP + 4 mM- Mg^{++} , pH 7.4.

Effects of cysteine. CySH was found to bring about definite activation of both pyrophosphatase and ATP-ase (Fig. 7), the maximum effect being produced with a concentration of 5–12 mM. Alkaline phosphatase, like that of some other tissues, was strongly inhibited by CySH, but acid phosphatase was uninfluenced.

Effects of tissue concentration. Fig. 8 shows the effects of tissue concentration. Good proportionality was obtained with the phosphomonoesterases at concentrations up to 8.5 mg./ml. for alkaline phosphatase and 7.5 mg./ml. for acid phosphatase. With pyrophosphatase and ATP-ase the inclusion of CySH as a co-activator is important from the point of view of enzyme assay; in its absence, a non-linear tissue concentration curve was obtained, but the linear relationship was restored when CySH was present. This phenomenon is possibly due to a protective action against partial deactivation by oxidation.

Progress of hydrolysis. Rates of hydrolysis remain constant in each case for periods of time well outside the selected times used in the assay. For ATP-ase, the relationship holds up to 30% decomposition, and for pyrophosphatase up to 60% decomposition, of substrate. The phosphomonoesterase activities, though not studied up to the maximum period of

constant rate, are constant up to a stage which covers the assay conditions.

Effects of various substances on acid phosphatase. Since the acid phosphatases of different tissues respond differently to certain inhibitors, it was of interest to test the action of some of these inhibitors on the adrenal cortex enzyme. The substances tried were selected from those used by Abul-Fadl & King (1949) in the differential characterization of plasma

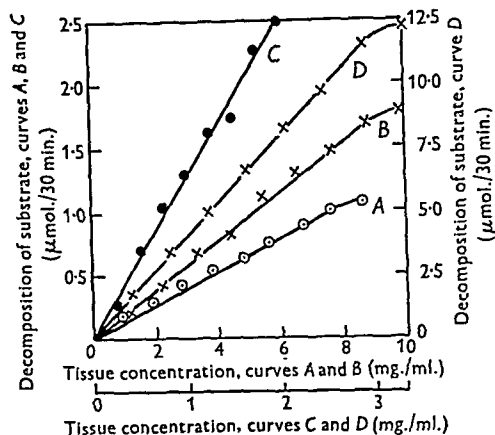


Fig. 8. Effects of tissue concentration on phosphatase activity of rat adrenal cortex at 37°. A, 19 mM-sodium glycerophosphate, pH 5.2; B, 6 mM-sodium glycerophosphate, pH 9.6; C, 1 mM- $Na_4P_2O_7$, in presence of 8 mM- Mg^{++} + 10 mM-CySH, pH 7.4; D, 3 mM-ATP in presence of 4 mM- Mg^{++} + 10 mM-CySH, pH 7.4.

acid phosphatases. Results are shown in Table 2. The behaviour was to some extent similar to that of the red-cell acid phosphatase. Ethanol and formaldehyde inhibited substantially; copper sulphate

Table 2. Effects of various substances on acid phosphatase activity of rat adrenal cortex at 37°

(Acetate buffer, pH 5.2. Sodium glycerophosphate (18 mM) as substrate. In the case of ethanol the tissue was pre-incubated with 0.4 vol. ethanol before making up the incubation mixture; otherwise the inhibitors were added directly to the incubation mixture.)

Added substance	Concn. (mM)	Inhibition (%)
$FeSO_4$	10	0
$CuSO_4$	0.2	86
Formaldehyde	170	30
Formaldehyde	600	73
Ethanol	—	68
Sodium DL-tartrate	—	91

in low concentration also caused strong inhibition, but ferrous sulphate was without effect. On the other hand, the enzyme was strongly inhibited by DL-tartrate, which does not affect the red-cell enzyme (Abul-Fadl & King, 1949).

DISCUSSION

The investigations indicate the general behaviour of the esterase and phosphatase reactions of the adrenal cortex, though the role of these systems in adrenal cortical function must clearly require much more detailed and extensive studies.

It seems doubtful whether the term 'lipase' can be applied to the tributyrinase action of adrenal cortex homogenates, in view of the apparent inactivity towards triolein, and the different response shown to bile salts as compared with pancreatic lipase. Since the earlier reports on the existence of an adrenal cortical lipase were based on its activity towards tributyrin, the point only involves a question of nomenclature. Since the enzyme bears a closer resemblance, with respect to substrate specificity, to the esterase of human plasma (cf. Adams & Whittaker, 1949), the term 'aliphatic esterase' would be the more appropriate.

The influence of various inhibitors on the aliphatic esterase and cholinesterase activities suggests that choline esters are hydrolysed by an enzyme system which is distinct from the aliphatic esterase, though the latter is present in so much greater strength. The results on cholinesterase activity are not easy to interpret. Although acetyl- β -methylcholine is attacked much more readily than benzoylcholine, the designation of 'true' or 'specific' cholinesterase is contra-indicated by the anomalous behaviour towards the selective inhibitors used. Further light on this matter would perhaps be thrown by experiments using a wider range of inhibitors. It seems possible in any case that cholinesterase plays a less important role in the cortex than in the medulla. It has been reported (Lange-mann, 1942) that the enzyme is present in much greater strength in the medulla than in the cortex; this was confirmed during the course of the present work by a few rough measurements (not reported) on pooled separated medullae, which indicated reaction rates approximately three times as high as for cortex. This distribution may be connected with the relative distribution of post-ganglionic sympathetic nerve fibres in the gland.

The properties of the adrenal cortical phosphatases, especially in regard to their pH optima and

the manner in which they are influenced by magnesium ions, are broadly similar to those of other tissues. ATP-ase and pyrophosphatase show characteristics similar to those of the corresponding water-extractable enzymes of other animal tissues; ATP-ase is activated by calcium and magnesium ions (cf. Frank, Lipschitz & Barth, 1950), the latter being the more effective, while pyrophosphatase is only active in presence of magnesium ions (cf. Naganna & Menon, 1948; Gordon, 1950).

At the present stage, perhaps the most important result arising from the investigations just described is the demonstration that, with the exception of cholinesterase, the levels of activity of all the enzyme systems concerned may be assayed in quantities of cortical tissue obtainable from the adrenals of an individual rat, so that the investigation of changes taking place under various abnormal conditions may be readily undertaken.

SUMMARY

1. Homogenates of demedullated rat adrenals show greater esterase activity with triacetin and tributyrin than with aliphatic esters of primary alcohols, and are inactive towards triolein. Choline esters are hydrolysed much less readily than tributyrin.

2. Bile salts and copper sulphate inhibit the aliphatic esterase. Eserine, the dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide (Nu 683) and *N*-diethylaminoethylphenothiazine (2987 R.P.), but not the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250), inhibit the cholinesterase.

3. The phosphomonoesterase, pyrophosphatase and adenosinetriphosphatase activities of such homogenates have also been studied. Acid phosphatase is inhibited by copper sulphate, formaldehyde, ethanol and DL-tartrate, but not by ferrous sulphate.

I wish to record my indebtedness to Mr F. E. Badrick for valuable technical assistance, to Dr M. Reiss, the Head of the Department, for his interest in the subject, and to Dr F. Bergel, of Messrs Roche Products Ltd., for supplies of the synthetic compounds Nu 683 and Nu 1250.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen. 1

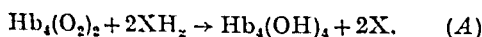
BY P. GEORGE AND C. J. STRATMANN
Department of Colloid Science, University of Cambridge

(Received 19 July 1951)

The study of the oxidation of haemoglobin and myoglobin to methaemoglobin and metmyoglobin by molecular oxygen is important in understanding the stability of oxyhaemoglobin and oxymyoglobin.

Brooks (1931, 1935) investigated the oxidation of haemoglobin (Hb) to methaemoglobin and found that under constant conditions of oxygen pressure, pH, salt concentration and temperature the reaction was first order with respect to the concentration of unoxidized haemoglobin, and that the rate of oxidation had a well defined maximum value at a low oxygen pressure (about 20 mm. at 30°).

Legge (1942) suggested the spontaneous decomposition of one of the oxygenation intermediates $\text{Hb}_4(\text{O}_2)_2$ as a reaction mechanism to account for these results. Later Lemberg & Legge (1949) amplified this mechanism by assuming the participation of two oxidizable XH_2 groups on the protein to make possible an intramolecular reaction fitting the stoicheiometric relation:



George (1949) put forward an alternative representation of the kinetics and discussed a possible intermolecular free radical mechanism which avoids some difficulties inherent in an intramolecular mechanism (George, 1952).

An investigation of the oxidation of myoglobin should help in deciding between mechanisms of this kind, for, since myoglobin has only one haem group per molecule, a reaction of type A occurring in this system should necessarily be second order.

This paper deals with the general chemistry of the reaction—choleoglobin and denatured protein formation—the overall stoicheiometry and the results of a kinetic study to establish this point.

In this paper the symbols Mb, MbO_2 , MetMb are used to represent reduced myoglobin, oxymyoglobin and metmyoglobin respectively. Similarly, in the case of haemoglobin, the corresponding symbols Hb, HbO_2 and MetHb are used.

MATERIALS AND METHODS

Preparation of pure, recrystallized equine metmyoglobin. The method employed is based on that of Theorell (1932) and Lawrie (1951). Four hearts were usually processed. After machine-mincing, the muscle tissue was extracted with water, the solution neutralized and treated with 1/5 of its volume of saturated, basic lead acetate to remove muscle globulins and other impurities. After centrifugation, the solution was again neutralized and sufficient solid Na_3PO_4 to remove excess lead was added. The pH was once more returned to 7.0 by addition of solid NaH_2PO_4 and the solution was centrifuged. It was found particularly important to adjust the pH to 7.0 at the points indicated above in order to obtain the maximum yield. It was also desirable to keep the temperature as near 0° as possible throughout the preparation. The MetMb solution was further purified by crystallization, induced by saturation of the solution with ammonium sulphate, followed by solution in 3M-phosphate buffer of pH 6.8. This treatment separates the MetMb from any traces of haemoglobin which may be present since haemoglobin is almost completely insoluble in phosphate buffer of this molarity (Morgan, 1935).

Lastly, the material was dissolved in the desired buffer solution (see below), traces of $(\text{NH}_4)_2\text{SO}_4$ were removed by

dialysis and the material stored at 0°. The yield averaged 1.1 g. MetMb/kg. of heart. The concentration and purity of the sample were determined by the method described by De Duve (1949).

Myoglobin solutions. The myoglobin solutions were buffered heavily as described by Brooks (1935) with K_2HPO_4 and KH_2PO_4 , the total concentration of phosphate being 0.6M and the $HPO_4^{2-}/H_2PO_4^-$ ratio 0.159/0.841. This gives the pH as 5.69 at 30°. The use of the heavily buffered solution has the advantages of preventing both microbial contamination and change of pH during the experiment. The latter is particularly important because the oxidation rate is dependent on the H ion concentration.

Before each experiment MetMb was converted into MbO_2 by first reducing to the ferrous form by addition of a small quantity of sodium hydrosulphite. Excess hydrosulphite is rapidly oxidized by atmospheric oxygen when the flask is gently shaken, and MbO_2 is formed.

Methods of analysis. Spectrophotometric methods of analysis were employed for the determination of MbO_2 and MetMb. The instrument used was a Unicam quartz spectrophotometer. Using a wavelength of 575 m μ , the millimolar extinction coefficient (E_{mm}) was very near to 11.0 for MbO_2 and 3.0 for MetMb at pH 5.69. These values varied slightly for different preparations. The solutions obeyed Beer's Law. At the beginning of each experiment the optical density of the pure MetMb solution was recorded. The MetMb was then converted into MbO_2 and the optical density recorded.

The fraction of MbO_2 in a given solution containing a mixture of MbO_2 and MetMb was determined by comparing the optical density of the solution with the corresponding optical densities of the two solutions containing 100% MbO_2 and 100% MetMb. It was found most convenient to work with optical densities between 0.6 and 0.8 for MbO_2 with the corresponding densities of 0.16 and 0.22 for MetMb. When using solutions of higher concentrations than would give values of this order of magnitude, appropriate dilution was made before reading in the spectrophotometer.

Apparatus and experimental method. MetMb solution (15–20 ml.) at the desired dilution was placed in the reaction flask, which was boat-shaped like those used by Meldrum & Roughton (1934), but without the partition along the bottom. The flask was placed in an ice bath and a minute quantity of sodium hydrosulphite added. The solution was gently shaken until the Mb was converted into MbO_2 . All oxidation experiments were carried out in air at 30° in 0.6M-phosphate buffer, pH 5.69, using the following procedure:

The solution (5 ml.) was withdrawn from the flask by means of a pipette, the optical density measured, and the solution returned to the flask. The flask was then transferred to the thermostat at 30°. The starting point of the reaction was taken at the time when the flask was placed in the thermostat. The flask was shaken mechanically 40 times/min., producing a small wave moving backwards and forwards across the surface of the solution, thus ensuring efficient stirring and renewal of the surface. At intervals 5 ml. samples of the solution were removed from the reaction flask, the optical density measured and the solution quickly returned to the flask. When using more concentrated solutions a smaller sample was removed and the appropriate dilution made before measuring the optical density. Results were recorded as percentage MbO_2 in the mixture from the time of the start of the experiment.

Warburg manometers were used in measuring the consumption of O_2 during the autoxidation in air; $1.3 \times 10^{-3}M$ solutions of myoglobin were used and the absorption of O_2 measured after the flask had been equilibrated. Calculations showed that no significant decrease in the O_2 pressure occurred as a result of the observed O_2 absorption. The same technique was used to measure the evolution of O_2 when MbO_2 was acidified with 1.0M-metaphosphoric acid and HCl.

Cholemyoglobin formation. Under the conditions of the experiments there was the possibility that cholemyoglobin might be formed. In order to test for this the following method was employed: two samples of myoglobin solution were taken, one immediately before an oxidation experiment was begun and the other 3 hr. later. Each sample was converted into the CO-haemochromogen by the method described by Lemberg, Legge & Lockwood (1941) and the spectrum examined over the range 500–650 m μ .

Denaturation check. In order to determine whether any denaturation of the protein occurred during the reaction, use was made of the reaction between MetMb and NaN_3 . On the addition of NaN_3 to a MetMb solution it is converted into the azide-metmyoglobin compound, the spectrum of which has two strong but diffuse bands at 575 and 542 m μ . Since only the ferric derivative of the native protein forms a complex with azide (Keilin, 1936) any change in the spectrum of the azide compound would be indicative of some change in the protein. Metmyoglobin solution (5 ml.) at pH 5.69 was treated with excess NaN_3 and the spectrum examined over the range 500–650 m μ . A separate 15 ml. sample of the same solution was converted to MbO_2 and allowed to oxidize as above for 3 hr. At the end of this time the material was oxidized fully by the addition of a small quantity of potassium ferricyanide. Azide was then added and the spectrum examined as before.

RESULTS

Choleglobin and denaturation check

In 3 hr. 60% of metmyoglobin was formed from oxymyoglobin. Using the methods described above it was shown (Figs. 1 and 2) that no detectable amount of either choleglobin or denatured protein is formed.

Oxidation kinetics

The metmyoglobin formation was measured at 10 and 20 min. intervals for four initial concentrations of oxymyoglobin: 0.296, 1.18, 2.11 and $2.96 \times 10^{-4}M$. Fig. 3 shows that the percentage oxidation in a given time is independent of the absolute concentration of myoglobin, showing that the oxidation is first order with respect to unoxidized myoglobin. A typical first order plot for the experiment when the initial concentration of myoglobin was $1.18 \times 10^{-4}M$ is given in Fig. 4, and Table 1 shows the first order constant obtained in the other experiments.

The mean value of the first order constant is $0.325 \pm 0.015 \text{ hr.}^{-1}$.

The rates of oxidation were reproducible using the same stock solution of myoglobin, but a certain amount of variation was observed between different

preparations of myoglobin and when the sample had been recrystallized. Table 2 gives values of k for seven different samples of myoglobin obtained in the course of 18 months' work. These values show a variation of some 7% which is to be compared with an uncertainty of about 5% in the value of an individual constant obtained from the first order plot.

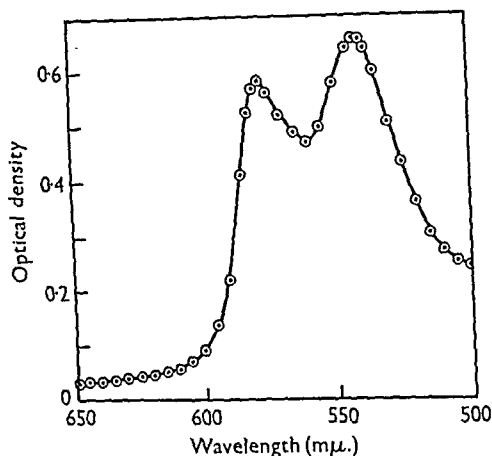


Fig. 1. Spectra of the carbon monoxide-protohaemochromogen complex formed before and after autoxidation in air at 30° in 0.6M-phosphate buffer of pH 5.69. —, from 5.1×10^{-5} M-MetMb; ○, from a solution of the same concentration after autoxidation for 3 hr.

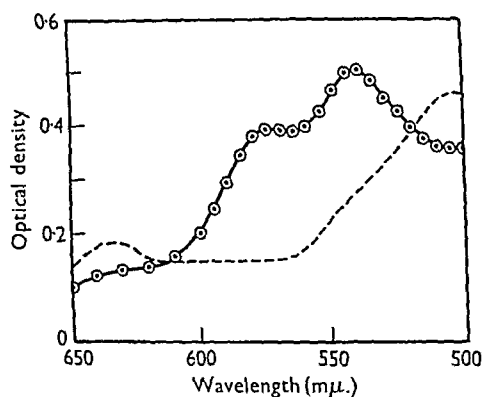


Fig. 2. Spectra of the azide-metmyoglobin complex formed before and after autoxidation of myoglobin in air at 30° in 0.6M-phosphate buffer of pH 5.69. —, from 5.1×10^{-5} M-MetMb; ○, from a solution of the same concentration after autoxidation for 3 hr.; ---, spectrum of acid metmyoglobin at the same concentration.

The variation between the several samples is not so marked as that found by Brooks (1931), working on the oxidation of haemoglobin, using laked blood, when in air at 25°, 0.2M-phosphate buffer solution of pH 6.29, $k = 1.54 \pm 0.28 \times 10^{-2}$ hr.⁻¹, i.e. an 18% variation. This comparison suggests that the variation may be due, in part, to the presence of other oxidizable substances present to a lesser

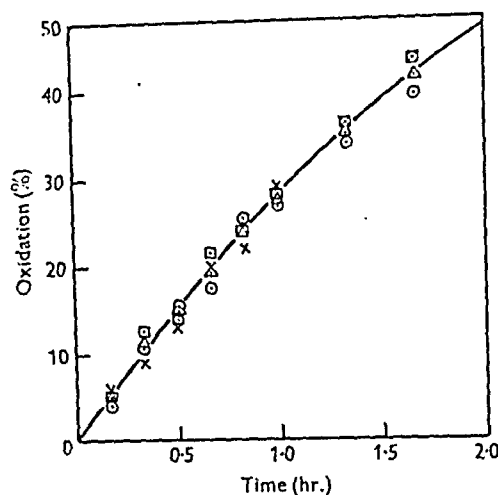


Fig. 3. The percentage formation of metmyoglobin during the autoxidation in air at 30° in 0.6M-phosphate buffer of pH 5.69 of four solutions of different initial concentrations of MbO₂. The points ○, △, □ and × refer to solutions of 0.296 , 1.18 , 2.14 and 2.96×10^{-4} M-myoglobin respectively.

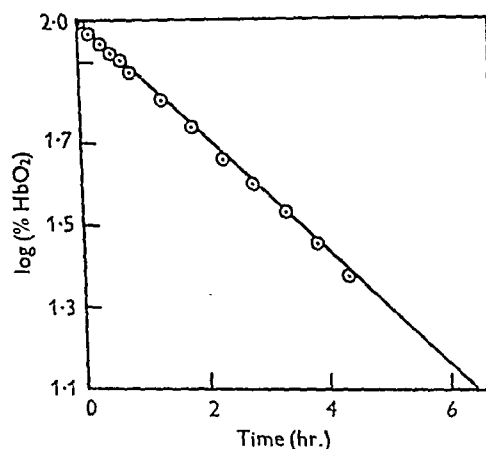


Fig. 4. A first order plot for metmyoglobin formation from spectrophotometric measurements. Initial concentration of MbO₂, 1.18×10^{-4} M.

Table 1. First order rate constant for the oxidation of myoglobin to metmyoglobin at four different initial concentrations of unoxidized myoglobin, in air at 30° in 0.6M-phosphate buffer, pH 5.69

(Spectrophotometric methods of analysis.)

Initial concentration of unoxidized myoglobin (mol. $\times 10^{-4}$ /l.)	First order rate constant k (hr. ⁻¹)
2.96	0.34
2.11	0.34
1.18	0.32
0.296	0.30
Mean	0.325 ± 0.015

degree in the purified myoglobin preparations used in the present experiments. This will be discussed later in relation to the results obtained in the next section.

Table 2. *First order rate constant for the oxidation of myoglobin to metmyoglobin in air at 30° in 0.6M-phosphate buffer, pH 5.69, using several samples of myoglobin*

(Spectrophotometric methods of analysis.)

No. of sample	First order rate constant k (hr. ⁻¹)
2*	0.32
3	0.38
4	0.26
4†	0.24
5	0.24
5†	0.28
6	0.25

Mean 0.27 ± 0.02

* Mean of four determinations.

† Recrystallized sample.

Stoichiometry of the autoxidation

Measurements of the oxygen absorption of small quantities of 1.3×10^{-3} M-myoglobin, in air at 30° in 0.6M-phosphate buffer, pH 5.69, using Warburg manometers showed that about 1.5 moles oxygen are absorbed for each mole metmyoglobin formed. Typical results are shown in Table 3. The final volume of oxygen absorbed was estimated by extrapolation, with a probable error of about 5%. The reliability of this extrapolated value is supported by plotting the absorption data as a first order reaction, taking this value as the end point. A linear plot is obtained as shown in Fig. 5 for the data relating to Exp. 2 in Table 3; the first order constant of 0.29 hr.^{-1} is in good agreement with the value obtained by spectrophotometric measurements of the metmyoglobin formed. The reproducibility of these oxygen absorption experiments was not particularly good. Exp. 2, using 2 ml. of solution, gave oxygen uptakes of 106, 70 and 90 $\mu\text{l.}$ when repeated, and the uncertainty in the ratio moles oxygen absorbed/moles metmyoglobin formed is thus 0.3. These absorption values were unaffected if the centre cup of the Warburg flask did not contain sodium hydroxide. It therefore appears that no carbon dioxide is evolved in the reaction. Even though the

total quantity of oxygen absorbed varies between these rather wide limits the first order constant obtained by the method given above (Fig. 5) was still identical with that found in the spectrophotometric measurements. No significant difference was observed in either the total quantity of oxygen absorbed or the first order constant when the flask was filled with oxygen or with air.

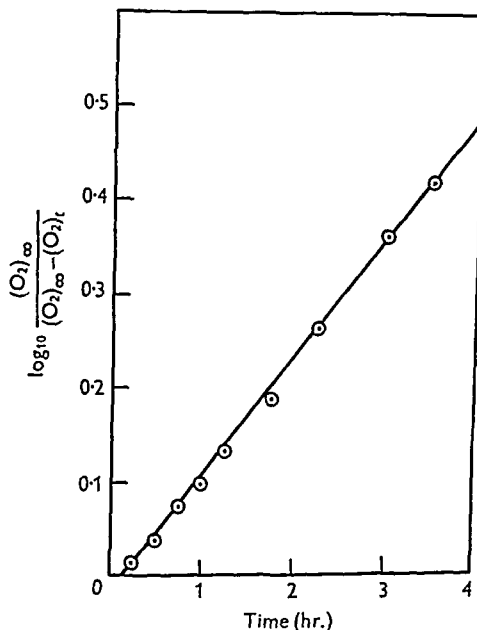
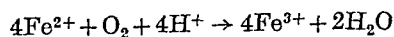


Fig. 5. First order plot for metmyoglobin formation from oxygen absorption data. Initial concentration of MbO₂, 1.3×10^{-3} M.

For myoglobin the half saturation pressure of oxygen is of the order 1–2 mm. and so in air about 99% is present as oxymyoglobin. If the stoichiometry corresponded to the hypothetical figure for the oxidation of the ferrous compound



three-quarters of the bound oxygen in oxymyoglobin should be evolved on autoxidation. The measured absorption of 1.5 ± 0.3 moles oxygen for each mole of metmyoglobin formed means that this quantity of oxygen is used up in the reaction in addition to all the bound oxygen originally present as oxymyoglobin and the stoichiometry for the autoxidation is thus

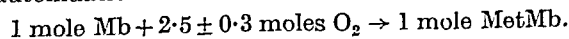


Table 3. *The oxidation of 1.3×10^{-3} M-myoglobin to metmyoglobin in Warburg flasks in air at 30° in 0.6M-phosphate buffer of pH 5.69*

Exp.	Volume of solution (ml.)	Oxygen absorbed (extrapolated value) ($\mu\text{l.}$)	Mol. O ₂ absorbed / Mol. MetMb formed
1	4.5	210 ± 15	1.60 ± 0.11
2	2.0	90 ± 5	1.54 ± 0.08

The overall chemical equation for the autoxidation will be given by this expression or some multiple of it.

Comparable data are not available for haemoglobin, but Roaf & Smart (1923) have shown that when oxyhaemoglobin is denatured by acidification about 50% of the bound oxygen is liberated. It appears that the remainder is used in oxidizing the haem to haemin and oxidizing hydrogen donor groups on the protein part of the molecule (Lemberg & Legge, 1949).

Acidification of oxymyoglobin, in the Warburg apparatus, with 1.0M-metaphosphoric acid gave no measurable evolution of oxygen, and with 1.0N-hydrochloric acid only 11% of this bound oxygen was released.

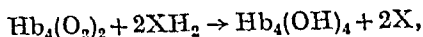
DISCUSSION

The oxidation of myoglobin to metmyoglobin by oxygen at pH 5.69 in the absence of acceptors is a relatively simple reaction. In contrast to the experiments of Lemberg *et al.* (1941), on the reaction of oxyhaemoglobin with ascorbic acid, no detectable amount of choleglobin is formed, and unlike the reaction which occurs when acid or pyridine is present, where denaturation accompanies oxidation, the protein remains in a native condition.

The results of the stoichiometric experiments do, however, suggest that the protein is not in its original state, for 2.5 ± 0.3 moles oxygen are used up for each mole of metmyoglobin formed. The simplest stoichiometry for the autoxidation of a ferrous compound involves four equivalents of ferrous compound for each oxygen molecule; thus additional reactions absorbing oxygen accompany the oxidation of the haem in myoglobin. The similarity between the kinetics of oxygen absorption and metmyoglobin formation argues against this oxygen being used to oxidize traces of impurity, and suggests that hydrogen donor groups on the protein molecule itself are involved. In this respect the experiments of Mirsky & Anson (1936) are particularly interesting. They showed that tyrosine and tryptophan groups in proteins were oxidized by even mild reagents like potassium ferricyanide, and that with tyrosine itself one mole could react with at least 2.6 moles ferricyanide. Myoglobin contains two molecules each of tyrosine and tryptophan per molecule (Tristram, 1949). There are thus, in the myoglobin molecule, four known groups susceptible to oxidation, and if the mechanism for the oxidation of the haem involves intermediates which are strong oxidizing agents it is possible that other amino-acid residues could also be attacked.

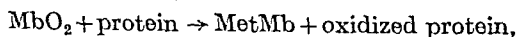
The participation of hydrogen-donor groups on the protein in the autoxidation mechanism has been suggested by Lemberg & Legge (1949), but for a

different reason. Legge (1942) showed that over a certain range of oxygen pressures Brooks's data on haemoglobin autoxidation (1931, 1935) could be explained if the reaction involved the spontaneous decomposition of the oxygenation intermediate $\text{Hb}_4(\text{O}_2)_2$ in an intramolecular reaction. To account for this, Lemberg & Legge postulated two oxidizable XH_2 groups in the haemoglobin molecule to complete the stoichiometry in an equation



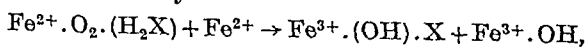
thus accounting for the full oxidizing capacity of the two oxygen molecules. The probable participation of hydrogen-donor groups on the protein, suggested by our experiments on oxygen absorption during the autoxidation of myoglobin, leads to a conclusion opposite to that of Lemberg & Legge. Since myoglobin contains only one haem group per molecule the consumption of 2.5 moles of oxygen for each mole of metmyoglobin formed must mean that the reaction is intermolecular.

It is clear that the production of metmyoglobin under the above conditions is not a straightforward autoxidation of a ferrous compound, but is a coupled oxidation. However, it differs in three important respects from the coupled oxidations studied by Lemberg *et al.* (1941). First, it does not appear to involve an added hydrogen donor like the ascorbic acid in Lemberg's experiments. Secondly, the metmyoglobin is not reduced by the hydrogen donor as methaemoglobin is reduced by ascorbic acid, and, as a consequence, there is no catalytic cycle for the continued oxidation of the hydrogen donor. Thirdly, whereas the coupled oxidation of ascorbic acid by oxyhaemoglobin is initiated by a direct reaction between these two molecules (Lemberg *et al.* 1941), the kinetics of myoglobin autoxidation, shown above to be first order in unoxidized myoglobin, do not support a reaction of the kind:



for, although this reaction could lead to a first order disappearance of myoglobin, the observed first order constant would not be independent of the absolute concentration.

By analogy with the mechanism put forward for haemoglobin autoxidation, Lemberg & Legge suggested that, in the case of myoglobin, the mechanism may be



and as a consequence the reaction would be second order in unoxidized myoglobin. The first order kinetics established above show that a mechanism of this type does not occur. The similarity that is now revealed between the kinetics of the autoxidation of myoglobin and haemoglobin casts doubt on the intramolecular mechanism in the case of haemoglobin, for it seems reasonable to expect that molecules so

alike in their general chemistry should react in the same way.

George (1949, 1952) discussed a free radical mechanism for haemoglobin autoxidation based on the stoichiometric relations $4Mb \equiv O_2$ or $2Hb \equiv O_2$ which would account for the observed kinetics in the case of haemoglobin. A general mechanism of this kind needs revising, since it has now been shown that oxidation of hydrogen-donor groups is likely to be involved.

SUMMARY

1. The autoxidation of myoglobin to metmyoglobin in air at 30° in 0.6M-phosphate buffer of pH 5.69 was studied at several different concentrations and using five preparations of recrystallized myoglobin.

2. The reaction was shown to be first order with respect to unoxidized myoglobin and the mean value of the first order rate constant was $0.325 \pm 0.015 \text{ hr.}^{-1}$.

3. Both spectrophotometric and oxygen absorption methods of analysis were used. The first

order rate constants obtained by both these methods were in good agreement.

4. Under the conditions of the experiments it was shown that no detectable denaturation of the protein or choleglobin formation occurred.

5. Measurement of the oxygen absorption of the reaction showed that 2.5 moles of oxygen were used for each mole of metmyoglobin formed, strongly suggesting that hydrogen donor groups on the protein molecule are involved.

6. These results are discussed, and it is concluded that in contrast to the intramolecular mechanism of the reaction postulated by Lemberg & Legge (1949) the reaction must be intermolecular.

7. In view of the similarity of the kinetics of the autoxidation of myoglobin and haemoglobin, doubt is cast on the idea of an intramolecular mechanism in the case of the autoxidation of haemoglobin.

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The Microbiological Assay of 'Strepogenin' with *Lactobacillus casei*

By E. KODICEK AND S. P. MISTRY*

Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council

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The presence of a factor in partially hydrolysed proteins, called 'strepogenin' (Sprince & Woolley, 1944), which had a growth stimulating effect for certain bacteria, has been reported by a number of investigators (Woolley, 1941; Sprince & Woolley, 1945; Pollack & Lindner, 1943; Smith, 1943; Wright & Skeggs, 1944; Wright, Fruton, Valentik & Skeggs, 1950). Woolley (1946) suggested that the peptide-like growth factor was a derivative of glutamic acid.

* Present address: Department of Animal Science, Agricultural Experiment Station, University of Illinois, Urbana, U.S.A.

A serylglutamic acid peptide from enzymic digests of casein stimulated *Lactobacillus casei* (Verdier & Ågren, 1948; Ågren, 1949). However, a number of synthetic peptides containing glutamic acid were either inactive or had a negligible activity (Woolley, 1948; Krehl & Fruton, 1948).

Dunn & McClure (1950) suggested recently that the strepogenin activity may be due to an unspecific effect of peptides, since the organism may use an essential amino-acid more readily in the bound than in the free form. Peptides of amino-acids were shown to be used by bacteria (Riesen *et al.* 1947;

Simmonds, Tatum & Fruton, 1947; Krehl & Fruton, 1948), but no stimulatory response was obtained with synthetic di- and tri-peptides containing leucine, valine, glycine or alanine (Ågren, 1947, 1948).

Glutamine and glutathione have been reported to have strepogenin-like activity (Woolley, 1948). The former could be differentiated from strepogenin by its heat lability (Pollack & Lindner, 1943; Woolley, 1946, 1948). Furthermore, Wright & Skeggs (1944) pointed out that the activity of enzymic digests of casein could not be accounted for on the basis of their glutamine content.

The present experiments were undertaken to investigate the specificity of the strepogenin effect and its distribution in biological materials. A satisfactory assay technique was developed using a modified medium described for the assay of B-vitamins (Clegg, Kodicek & Mistry, 1952). For preliminary communication, see Mistry & Kodicek (1951).

MATERIALS AND METHODS

Strepogenin standard (IA37°). A partial hydrolysate of insulin instead of the Wilson liver fraction L (Sprince & Woolley, 1944, 1945) was used as the strepogenin standard since it was shown to be a rich source of this growth stimulatory factor. In preliminary trials the treatment described below was found to liberate the maximum activity. Digestion for a longer period or at lower temperature resulted in decreased activity.

Table 1. *Basal medium for the assay of strepogenin activity*

Constituent	Amounts/10 ml. single strength medium
Casein (Labco), H ₂ SO ₄ -hydrolysed*	50 mg.
Glucose	300 mg.
Potassium acetate	300 mg.
DL-Alanine	1 mg.
L-Asparagine	1 mg.
L-Cystine	2 mg.
DL-Tryptophan	2 mg.
Adenine, guanine, uracil and xanthine: each	0.1 mg.
Aneurin chloride hydrochloride	10 µg.
Riboflavin	10 µg.
Nicotinic acid	10 µg.
Pyridoxin hydrochloride	25 µg.
Calcium-D-pantothenate	10 µg.
p-Aminobenzoic acid	2 µg.
Biotin (free acid)	0.04 µg.
Pteroylglutamic acid	0.1 µg.
K ₂ HPO ₄ and KH ₂ PO ₄ : each	25 mg.
Salt solution E*	0.05 ml.

* Clegg *et al.* (1952).

200 mg. crystalline bovine insulin (Boots Pure Drug Co. Ltd.) were hydrolysed for 3 hr. at 37° in 5 ml. of conc. HCl. The acid was carefully removed under reduced pressure on a water bath and the solids dissolved in about 45 ml. of water. The pH was adjusted to 6.8-7.0 with 40% KOH, the volume made up to 50 ml., and the solution filtered and stored at 4° under toluene. Its concentration will be referred to in terms

of the original weight of insulin. This preparation was assigned a potency of 100.

Basal medium. The medium previously described (Clegg *et al.* 1952) was modified by omitting Norite-treated peptone and adding L-asparagine (Table 1).

Bacteriological procedure. *Lb. casei*. A.T.C.C. No. 7469, was used throughout this study as the test organism. The maintenance of the culture and other bacteriological techniques have been described previously (Clegg *et al.* 1952).

The inoculum was standardized to contain 30 µg. of dry wt. of bacteria per ml. and one drop was seeded aseptically into 10 ml. medium. The seeding contained 1 µg. of dry wt. of bacteria, i.e. about 1×10^4 viable cells as determined by the plate count method.

The assay tubes were incubated at 37° for 18 hr. and the resulting growth was measured turbidimetrically against the corresponding blank in a single-cell photoelectric colorimeter, using a neutral grey filter. The results were calculated from a calibration curve for *Lb. casei* in terms of dry wt. bacteria.

Choice of the size of inoculum. It was pointed out by Sprince & Woolley (1944, 1945) that the use of a small inoculum was important in the assay of strepogenin, since with larger seedings the responses due to strepogenin additions were less marked. Similar results were obtained in the present study. The medium (10 ml.) was seeded with inocula of varying strength, increasing in geometric progression. Within a range of 0.275-1.1 µg. of inoculum, the ratio of the growth stimulation by strepogenin to the concentration of inoculum, remained constant. At higher concentrations, the blanks increased relatively more than the responses obtained with the strepogenin preparation. Therefore, an inoculum containing 1 µg. of dry wt. of bacteria was adopted.

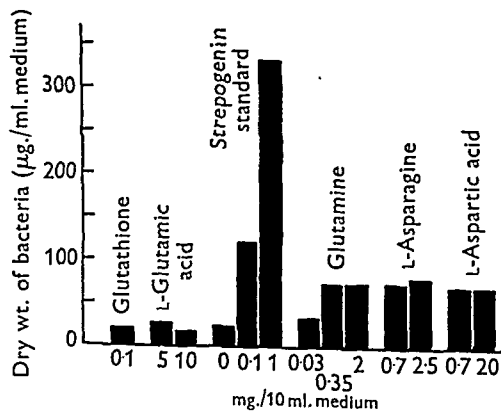


Fig. 1. Effects of various substances on the rate of growth of *Lb. casei* (18 hr. incubation) compared with the strepogenin standard, IA37°.

RESULTS

Effect of various substances on the growth of Lb. casei

A number of substances have been reported to stimulate the growth of *Lb. casei* and they were therefore reinvestigated with the use of the medium from which asparagine was omitted.

It will be seen from Fig. 1 that glutathione and glutamic acid had no stimulatory effect. Glutamine, added after autoclaving the medium, gave a small

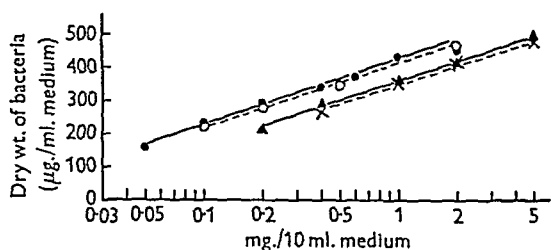


Fig. 2. Dose response curves of the strepogenin standard, IA37°, ●; insulin, Novo, free of hyperglycaemic-glycogenolytic factor, ○; trypsin, Armour, ▲; and plasma albumin, bovine, Armour, ×.

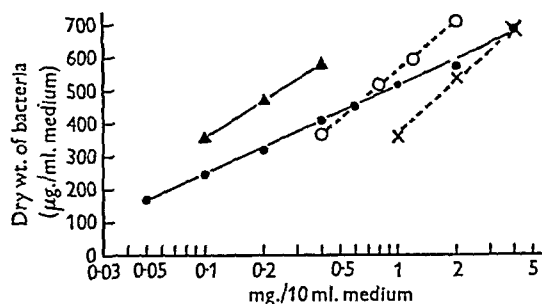


Fig. 3. Dose response curves of the strepogenin standard, IA37°, ●; insulin, sheep, Boots Pure Drug Co. Ltd., ▲; chymotrypsin, Armour, ○; tobacco mosaic virus, ×.

Table 2. *Strepogenin activity of insulin, liver and yeast preparations*

Material*	Relative strepogenin activity (%)
Insulin, bovine;† strepogenin standard, IA37°	100
Insulin, bovine;† unhydrolysed	11
Insulin, bovine;† different batch	112
Insulin, bovine;† recrystallized	95
Insulin, bovine;† hydrolysed with chymotrypsin	103
Insulin, pig†	412
Insulin, sheep†	400
Insulin, Novo, free of hyperglycaemic-glycogenolytic factor	90
Hepamino, proteolysed liver powder, Evans; unhydrolysed and hydrolysed	20†
Liver fraction L, Wilson; unhydrolysed and hydrolysed	2†
Marmite, yeast extract; unhydrolysed	6

* Except where mentioned, all materials were hydrolysed with conc. HCl for 3 hr. at 37° and tested at three or more levels.

† These preparations were obtained from Boots Pure Drug Co. Ltd.

‡ Values denote activity not strictly comparable to the strepogenin standard. Activities were calculated at 3.5 and 50 mg. per 10 ml. medium for Hepamino and liver fraction L respectively.

growth response. However, the response was considerably below that obtained with the strepogenin standard. Asparagine and aspartic acid also had a small stimulatory effect during the 18 hr. test period. In the medium used for assay, i.e. with asparagine

present, no further stimulation of growth was observed with either glutamine or aspartic acid. Kodicek & Mistry (1949) noted that the presence of asparagine greatly enhanced the effect of strepogenin preparations and therefore it was included in the medium. A detailed study of these effects will be reported in another communication.

Table 3. *Strepogenin activity of purified proteins*

Material*	Relative strepogenin activity (%)
Insulin, bovine, Boots Pure Drug Co. Ltd.; strepogenin standard, IA37°	100
Pepsin, Armour, 1:10000 potency; unhydrolysed	19
Pepsin, Armour, recrystallized, 1:60000; unhydrolysed	6
Pepsin, Armour, 1:10000	39
Pepsin, Armour, recrystallized, 1:60000	160†
Peptides 16P obtained on recrystallization of pepsin, Armour, 1:10000	17
Trypsin, Armour	48
Chymotrypsin, Armour	153†
Turnip yellow mosaic virus	86†
Tobacco mosaic virus	56†
Myosin, rabbit	104†
Lactoglobulin, crystalline	49†
Plasma albumin, bovine, Armour	45
Egg albumin	19
Crude protein from degraded wool	13
Globin, horse	10
Globin, human	9
Globin, cow	5
Haemoglobin, bovine, Armour	5
Pea globulin	4
Edestin	2
Gelatin	1
Salmin sulphate, British Drug Houses Ltd.	0
Casein, vitamin-free, Labco	5
Casein, vitamin-free A/E, Glaxo	2
Casein, vitamin-free LW, Glaxo	2
Casein, vitamin-free, Hopkin & Williams	2
Casein, hydrolysate, vitamin-free, Ashe; unhydrolysed	0.4
Casein hydrolysate, for oral administration, unhydrolysed	0

* Except where mentioned, all materials were hydrolysed with conc. HCl for 3 hr. at 37° and tested at three or more levels.

† Values denote activity not strictly comparable to the strepogenin standard.

With the modified medium (Table 1), a linear dose response was regularly obtained when the logarithm of the dose was plotted against the dry weight of bacteria (Figs. 2 and 3). The agreement between triplicate tubes within an experiment at all the levels tested was satisfactory, with a coefficient of variation less than 5%.

The method has been applied to the assay of the strepogenin activity in a number of highly purified proteins and biological preparations (Tables 2 and 3). The results were expressed as a percentage of the activity of the standard (IA37°). Since the method

of hydrolysis used for preparing the strepogenin standard was shown to liberate the maximum activity from the intact insulin molecule, the same procedure was adopted for the test materials. It is realized, however, that the activities may not be a true measure of the strepogenin content of materials, but represent only the activity liberated from proteins under comparable conditions. It may be that other methods of hydrolysis would yield higher values.

The materials were assayed usually at three or four levels. From the results, a logarithmic dose response curve was constructed and the relative potency was calculated from the linear dose response of the standard (IA 37°). If the slope of the dose response curve of the substance differed from that of the standard, the calculated activity varied at different levels. This is denoted in the tables by an asterisk and will be discussed below in more detail.

Insulin and liver preparations. The strepogenin activity of hydrolysed bovine insulins from different batches did not vary more than about 10%. Recrystallization of insulin or chymotrypsin digestion did not result in a further increase in potency. Unhydrolysed bovine insulin showed only negligible activity.

Sutherland, Cori, Haynes & Olsen (1949) have reported the separation of a hyperglycaemic-glycogenolytic (H-G) factor from amorphous and crystalline insulins. Insulin, Novo, which contains no H-G factor, had 90% of the strepogenin activity of the crystalline insulin standard. This indicated that the activity of the latter was not associated with the H-G factor.

The relative distribution of strepogenin activity in insulins showed certain species differences. The pig and sheep insulins were about four times as active as bovine insulin, but the greater activity could not be correlated with the differences in their amino-acid content (Sanger, 1949).

Wilson liver fraction L, and proteolysed whole liver powder, Hepamino Evans, tested up to levels of 50 and 4 mg. respectively, per 10 ml. medium, had a low activity (Table 2). At higher concentrations, however, their growth-stimulatory effect surpassed that of the strepogenin standard (Fig. 4).

Crystalline enzymes, viruses and other proteins (Table 3). Recrystallized pepsin and chymotrypsin 'Armour' when hydrolysed had a high activity of the order of 153-160%. Less pure samples were less active. In agreement with this finding, the peptides '16P', impurities obtained on purification of pepsin (Ingram, 1950), had also a negligible strepogenin content. The unhydrolysed enzymes showed very little activity (6-19%).

Turnip yellow mosaic virus and tobacco mosaic virus showed a high strepogenin content (56-86%) when hydrolysed, although the slopes of the dose response curves were different from that of the

standard. The unhydrolysed tobacco mosaic virus was only 6% active.

Myosin, lactoglobulin and plasma albumin were a good source of strepogenin activity, but the steeper response curves obtained with myosin and

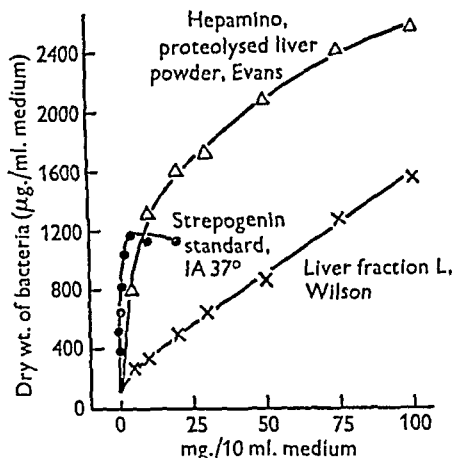


Fig. 4. Growth responses of liver preparations compared with the strepogenin standard, IA 37°.

lactoglobulin were not strictly comparable to the strepogenin standard. Other proteins, including casein, had a negligible potency.

DISCUSSION

In the present study a medium has been used which is fully supplied with known growth factors and any increase in the rate of growth should be due to unknown factors. In the presence of an optimum concentration of the strepogenin standard an increase in growth of as much as about 1000 µg. of dry weight of bacteria per 10 ml. medium was obtained within 18-20 hr. of incubation.

Under these conditions the strepogenin activity seemed to be a specific property of certain partially hydrolysed proteins and could not be replaced by the various substances tested such as glutamine, glutamic acid, glutathione, asparagine or aspartic acid.

Intact protein molecules such as insulin, pepsin and tobacco mosaic virus showed little or no activity. In keeping with its supposed peptide structure the strepogenin effect was only observed when the protein molecule was broken down by mild acid hydrolysis or chymotrypsin. In agreement with Sprince & Woolley (1945) the strepogenin activity was lost on prolonged treatment with HCl.

The results for the various proteins and biological materials could be classified into two groups. The first showed a growth response curve lying, within experimental error, parallel with that of the strepogenin standard (Fig. 2). In the second group a steeper dose response curve was obtained, which diverged significantly from the slope of the standard

(Fig. 3). It seems that in these cases factors other than those present in acid-hydrolysed insulin were effective. The highest potency compared to insulin was found in enzyme and virus proteins, myosin, lactoglobulin and plasma albumin. Casein had a low activity. The comparison of our data with those of other authors (Sprince & Woolley, 1945; Wright *et al.* 1950) is difficult because of the different techniques employed. The strepogenin activities of trypsin, casein, haemoglobin, gelatin and salmin agree with those found by Sprince & Woolley (1945).

Of the biological materials assayed the liver preparations showed growth effects characteristic of the second group which differed in its behaviour from that of the insulin preparation. At low concentrations they showed little activity but at high levels the responses surpassed the maximum obtained with the strepogenin standard. Their activity remained the same whether the liver fractions were hydrolysed or not. It seems that besides strepogenin, other factors are present in liver preparations which are considerably more potent in stimulating the growth of the organism. The effects may be similar to those reported by Stokes, Koditschek, Rickes & Wood (1949) and Verdier & Ågren (1948).

The factors seem to be different from vitamin B₁₂ which has no stimulatory effect on *Lb. casei*. Moreover, the Wilson liver fraction L was active, although it does not contain the anti-pernicious anaemia factor.

The identical activity of unhydrolysed and hydrolysed liver preparations would seem to exclude factors of peptide nature, unless such peptides are normally present in the unhydrolysed materials, as indeed has been shown by Borsook *et al.* (1949), who isolated a peptide 'A' from unhydrolysed homogenates of liver.

The stimulatory effect of strepogenin preparations, observed during the early growth period, is most likely due to the shortening of the lag period. Whether the response given by the insulin prepara-

tion is due to one specific substance or a number of substances is difficult to decide. It seems that the stimulatory effect of partially hydrolysed proteins is more specific than suggested by the unspecific peptide theory of Dunn & McClure (1950). This would not explain the great differences in activities of proteins otherwise closely related. On the other hand, these activities cannot be explained by the existence of a single factor, but indicate that the strepogenin effect may be due to certain peptides which have a particular configuration.

SUMMARY

1. A satisfactory procedure for the assay of strepogenin activity has been developed.

2. The method was applied to the assay of strepogenin in highly purified proteins. Insulins from various species differed in their activity. Liver preparations, as well as some of the pure proteins assayed, seem to contain a factor or factors considerably more potent than the insulin preparation used as the strepogenin standard.

3. The peptide(s) responsible for the strepogenin activity were shown to be an integral part of the protein molecule and not associated with an impurity such as the hyperglycaemic-glycogenolytic factor present in commercial preparations of crystalline insulins.

4. Glutamine, asparagine and aspartic acid showed a slight strepogenin-like activity; glutamic acid and glutathione were inactive. None of these substances could replace the strepogenin effect of partially hydrolysed proteins.

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Sedimentation and Diffusion of Human Albumins

1. NORMAL HUMAN ALBUMINS AT A LOW CONCENTRATION

By P. A. CHARLWOOD

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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The sedimentation and diffusion of normal human albumins have been studied as the initial part of a programme designed to investigate whether the molecular weights of the serum and urinary proteins in cases of nephrosis do differ from normal, as has been suggested by a few observers (e.g. Bourdillon, 1939). The present results are being communicated separately, as some delay appears likely before sufficient suitable pathological samples have become available for study.

Since the final object of this work is a comparison of normal and pathological materials, the limitations thus imposed have affected both the detailed technique and the scope of the experiments. Although it is well known that the albumin fraction isolated by electrophoresis contains at least two distinct components (e.g. Hoch & Morris, 1945), this method of isolation was selected, as the purpose was to determine the mean molecular weight of the albumin fraction. In any case no human albumin preparation yet obtained satisfies all criteria of purity. A particular advantage of the electrophoretic method in dealing with nephrotic sera is the possibility of separating albumin free from α -globulin, which is usually present in greatly increased amounts in such cases.

Sedimentation experiments require little protein, whereas, until recently, diffusion work needed appreciably more. The introduction of the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948; Ogston, 1949) has made it possible to obtain results with small amounts of material and much more rapidly. The limiting factors introduced by working with pathological samples are the small volumes of serum often obtainable and the low concentration of urinary protein. Electrophoretic isolation of more than a certain volume of protein solution (in this case 6 ml.) can be laborious and excessively time-consuming. It was, therefore, considered impracticable (often almost impossible) to prepare sufficient albumin to enable the values of $s_{20,w}^0$ and $D_{20,w}^0$ to be measured as a routine by extrapolation to zero concentration. Since the indications are that $s_{20,w}$ and $D_{20,w}$ vary rather slowly with moderate alterations in concentration so far as globular proteins are concerned, it was decided to make all measurements at a standard low concentration of 0.2–0.3%, when results should

all be comparable and should not differ very greatly from $s_{20,w}^0$ and $D_{20,w}^0$. All values of s are in Svedberg units and values of D in units of 10^{-7} cm.² sec.⁻¹.

EXPERIMENTAL

Samples of blood from normal individuals were collected without any preservative, allowed to stand overnight in the refrigerator, and the sera separated by low-speed centrifugation. After removal of the few residual red cells by centrifugation at a slightly higher speed, the serum was diluted with buffer to give a total protein concentration of about 2%, and dialysed in the cold against 2 l. of buffer for 2–3 days.



Fig. 1. Electrophoretic pattern of human serum albumin. Ascending (anode) limb. Migration is from left to right. Veronal buffer, pH 8.6, $I=0.05$. Protein 0.7%. Exposure after 56 min. at 6.3 V./cm.

Electrophoretic separations were carried out in the standard form of Tiselius apparatus at $+0.5^\circ$, the albumin boundary in the ascending limb being held stationary at a suitable level by means of an electrolytic compensator, as described by Johnson & Shooter (1949). Phosphate buffer (pH 8.0, $I=0.1$), used in the preliminary work, was found not adequately to resolve α_1 -globulin from albumin. A number of experiments were then carried out in veronal buffer (pH 8.6, $I=0.05$), but, since even here the resolution of α_1 -globulin may not be sufficiently good, the medium finally adopted was veronal buffer (pH 8.6, $I=0.1$). The maximum current considered safe, so far as heat convections were concerned, was about 20 ma. It was then possible after 6–10 hr. to recover from the ascending limb 3 ml. of solution containing

0.5–1.0% albumin. In order to check that no accidental contamination with globulin had occurred, every sample of albumin was examined in the micro cell of the Tiselius apparatus, a typical electrophoretic pattern being that shown in Fig. 1. About 4 ml. of solution, containing all the albumin, were removed from the micro cell. The concentration was calculated from the area of the peak in the diagonal schlieren pattern, the appropriate constants of the optical system being known. After dilution to bring the concentration into the selected range the albumin solution was dialysed for 5–6 days prior to sedimentation and diffusion. The few early preparations made in phosphate buffer were dialysed against the same buffer at this stage. Samples obtained in the veronal buffer of $I=0.05$ were dialysed against 0.2N-sodium chloride, but in the later work the sodium chloride concentration was reduced to 0.05N for the reason discussed later.

Sedimentation measurements. These were made in a Spinco ultracentrifuge, a short description of which has been given by Smith, Brown, Fishman & Wilhelmi (1949). The cell and

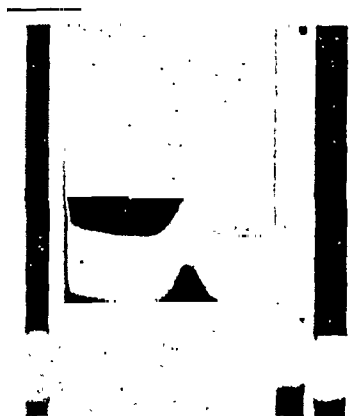


Fig. 2. Ultracentrifuge pattern of human serum albumin. Sedimentation is from right to left. Sodium chloride solution, 0.2N. Protein 0.2%. Exposure 71 min. after reaching full speed.

rotor are of conventional design and dimensions. The rotor spins in a high vacuum ($\sim 0.5 \mu$. of mercury) which is maintained by rotary oil and oil diffusion pumps. It is driven by an electric motor transmitting power to a special gear box (ratio 5:1) and a shaft which enters the vacuum chamber through an oil-seal of the type used in air-turbine machines. The rotor can be run at a series of set speeds, each of which is automatically maintained. Once the set speed is reached, variations are due almost entirely to fluctuations of mains frequency. A revolution counter, geared down in the ratio 6400:1, is provided which, together with a stop-watch, enables the mean speed during any interval of time to be determined accurately. A diagonal-bar schlieren optical system is incorporated, and photographic exposures can be taken either automatically at set intervals or manually. One such record is shown in Fig. 2.

The practice followed in these runs was to bring the rotor to full speed (60 000 rev./min.), to take the first photograph after about another 20 min. when the albumin peak had fully separated from the meniscus, and thereafter to take another four photographs automatically at 16 min. intervals.

As a routine the average speed of rotation was calculated from the counter readings immediately after the first and last photographs, but the speed during individual intervals could deviate from this. However, experiments in which the mean speed was also determined between each successive pair of photographs gave results almost identical. Further justification for the simpler procedure was provided by the small standard errors of the slopes of the $\log x-t$ lines (see Results). A final check on the accuracy of the counter was obtained by comparing its readings with the light impulses registered on an electronic counter fed from a photocell interposed in the optical system.

Travelling microscope measurements of the records (to the nearest 10μ .) gave the distance of the boundary (defined as the maximum of the symmetrical peak observed) from the index marks. The boundary was thus located with adequate sharpness, and the correctness of its position was checked by verification of the magnification of the optical system ($\sim 2:1$) over different parts of the field. The known distances of the index marks from the axis of rotation, which were measured several times, allowed the distance (x) of the boundary from that axis to be calculated at different times (t).

Before the chamber was shut the rotor temperature was recorded by means of a contact thermocouple with a reference junction immersed in an intimate mixture of ice and water in a Dewar vessel. The scale of the indicating galvanometer was carefully calibrated with the measuring couple at a series of temperatures registered by a standard thermometer. The rotor temperature at the end of these runs was usually $1.0-1.5^\circ$ higher than initially. The mean of initial and final rotor temperatures was taken as the temperature during the run (Pickels, 1950). In view of the importance of an accurate temperature estimation, both the contact thermocouple calibration and the standard thermometer were checked from time to time.

Possible variations in rotor temperature, due to different causes, were investigated as follows. The thermocouple leads were introduced into the vacuum chamber in such a way that the temperature of the (stationary) rotor could be observed whilst evacuation took place. This proved that no measurable change of temperature occurred during the process. It was also shown that no appreciable flow of heat took place along the driving shaft to (or from) the gear-box. Since the effect of any residual air in the chamber must be to raise the temperature of the moving rotor, the observed rise needs no other explanation. Low results (see Discussion) would make it necessary to postulate that the rotor temperature during the runs was actually lower than was believed. The only remaining possibility of error in estimating the temperature arises from the use of the refrigerating coils round the chamber to diminish or prevent the overall temperature rise in the rotor. If the refrigeration produced a drop in rotor temperature during the run, whilst admission of air to the chamber at the conclusion of the run caused a rapid rise, the estimated mean value would be too high. Although evidence against this hypothesis was quite decisive, as will be seen from the examples shown in Table I of duplicate experiments carried out with and without cooling, refrigeration has been dispensed with at present in order to eliminate any possibility of criticism on this point.

Diffusion measurements. The Gouy diffusometer used here was the model described by Creeth (1952), a version of the instrument developed by Coulson *et al.* (1948) and Ogston (1949). The technique followed was exactly that

described by Creeth (1952), the only points it is necessary to emphasize being as follows. With the dilute solutions used it is vital that the value of j_m (Longworth, 1947) for the dialysed protein solution should be measured with the greatest possible precision. Thus, it is obvious from the equation which describes the behaviour of mixtures (Ogston, 1949) that, should there be an excess or deficiency of salts in the protein solution (due to such causes as lack of dialysis equilibrium, or accidental dilution of the upper solvent layer through insufficient washing out of the diffusion cell), an incorrect result will follow. This is the reason for the ample time allowed for dialysis. To guard against evaporation, dialysis was carried out in closed vessels and the dialysis bags were not removed until immediately before the diffusion experiments. In some of the later work, as an additional precaution, the strength of the sodium chloride was reduced to 0.05N, a value still high enough to depress anomalies under the conditions of these experiments. Although dialysis took place in the refrigerator, for the last few hours the solutions were left at room temperature to allow dissolved air to be expelled. Otherwise the value of j_m might have been affected slightly (Gosting & Morris, 1949), and air bubbles were liable to form in the diffusion cell, interfering with the optics and rendering an experiment worthless.

The pipette holds sufficient solution for three or four runs, but experience has shown that the first run may give a spuriously high result, evidently through insufficient 'clearing' of the cell. After this initial run, which was not recorded, the boundary was sharpened again, and the next two runs were recorded. Generally there was enough material to repeat the whole procedure with a further pipette-full, giving four independent determinations with each solution.

In one case a stronger solution of albumin was prepared (0.9%) and diffusion carried out in 0.2N-sodium chloride solution. The resulting patterns contained a large number (33) of interference minima, the relative positions of which could be analysed to show whether or not the boundary involved an essentially Gaussian distribution.

Photography. Both electrophoretic and diffusion patterns were recorded on Ilford Thin Film Half Tone Panchromatic plates, the former being processed in a caustic-quinol developer, whilst for the latter 'Wellington borax' developer

was used in order to reduce the grain size. This borax developer was also used for the ultracentrifuge plates, which were Ilford Rapid Process Panchromatic.

RESULTS

Sedimentation experiments

The formula $s = \frac{\log_e x_2/x_1}{\omega^2(t_2 - t_1)}$ has often been used for calculating the sedimentation constant for successive time intervals (Svedberg & Pedersen, 1940). These are then corrected for temperature, etc. As the exact manner of the temperature variation of the rotor in the Spinco ultracentrifuge is not known, the mean value of s was worked out from the slope of the best straight line through the points on the graph of $\log x$ plotted against t (cf. Cecil & Ogston, 1948), ω and T being taken as the mean values for the whole run (see above). The standard error of the slope was always small (0.2–1.0 %). The sedimentation constants, all reduced to water at 20°, are shown in Table 1.

The degree of reproducibility, illustrated by preparations 2 and 3, was quite satisfactory, particularly in view of the fact that in the alternative refrigeration was used. Any real differences between the values of $s_{20,w}$ for different solutions due to concentration effects are estimated as at most 1%, and therefore less than the errors involved in the value, 4.25, for preparations made in 0.2N-sodium chloride (statistically just different from the mean value, 4.25, as this might have been due to a small error in the original calibration of the ultracentrifuge). The value of all results, 4.25, is therefore

Diffusion experiments

Mean values of the diffusion constants for each sample, reduced to water at 20°, are shown in Table 2. Except in the case of preparation 2

Table 1. Sedimentation constants of normal human serum

Serum no.	Buffer used in isolation	Solvent for sedimentation	$s_{20,w}$ (Svedberg units)	Standard error
1	Phosphate ($I=0.1$)	Phosphate ($I=0.1$)	4.25	0.10
2	Veronal ($I=0.05$)	0.2N-Sodium chloride	4.25	0.10
3			4.25	0.10
4			4.25	0.10
5			4.25	0.10
6			4.25	0.10
8	Veronal ($I=0.1$)	0.2N-Sodium chloride	4.25	0.10
9			4.25	0.10
10			4.25	0.10
11			4.25	0.10
12			4.25	0.10
13			4.25	0.10

* Refrigeration was used for preparations 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13.

Table 2. Diffusion constants of normal human serum-albumin preparations

Serum no.	Buffer used in isolation	Solvent for diffusion	Protein concn. (%)	No. of experiments	$D_{20, w}$
4	Veronal ($I=0.05$)	0.2 N-Sodium chloride	0.27	4	6.44
5			0.24	2	6.23
6			0.27	4	6.34
7			0.23	2	6.26
8	Veronal ($I=0.1$)	0.05 N-Sodium chloride	0.24	4	6.44
9			0.26	4	6.30
10			0.20	4	6.27
11			0.25	4	6.16
12			0.24	4	6.40
13			0.21	4	6.28

the same sample agreed within $\pm 2\%$, in accordance with the observations of Creeth (1952) at similar concentrations. The final mean of all measurements was 6.32. Although there was no correlation between diffusion constant and concentration, which was not surprising in such a narrow range and with relatively high errors for individual measurements, detailed statistical analysis showed that the differences between the various preparations were nevertheless just significant on the basis of a probability of 0.05; these differences were independent of the buffer used in isolating the samples.

Table 3. Diffusion of normal human serum albumin in 0.2N-sodium chloride; variation of C_t with j

(Time—approximately 10 min. after the beginning of diffusion. $j_m=33.59$.)

j	Y_j (cm.)	C_t (cm.)
0	0.9677	1.0700
1	0.8905	1.0677
2	0.8306	1.0685
3	0.7771	1.0676
4	0.7290	1.0670
5	0.6836	1.0646
6	0.6420	1.0634
7	0.6032	1.0625
8	0.5671	1.0627
9	0.5331	1.0639
10	0.4998	1.0633
12	0.4377	1.0630
14	0.3804	1.0632
16	0.3266	1.0621
18	0.2755	1.0577
20	0.2278	1.0523

The sample at 0.9% concentration gave for $D_{20, w}$ 6.14, 6.11, 6.06 and 6.08 (mean 6.10), reproducibility being much better than at the lower concentrations. However, the main point of these experiments was to obtain records for examination of homogeneity. Measurements were made at two different time marks on each record, at about 3 and 10 min. respectively after the beginning of diffusion. The equivalent treatments of Longworth (1947), as modified by Gosting & Morris (1949), and Ogston (1949) were applied. Typical results are shown in

Table 3. The values of C_t (defined by Longworth, 1947) show a pronounced, but fairly small, tendency to decrease as j increases, proving that there is some departure from the ideal Gaussian form.

DISCUSSION

Sedimentation constants

The trend of results in the Spinco ultracentrifuge has been mentioned in a recent paper from this laboratory (Creeth, 1952). In Fig. 3 the results for

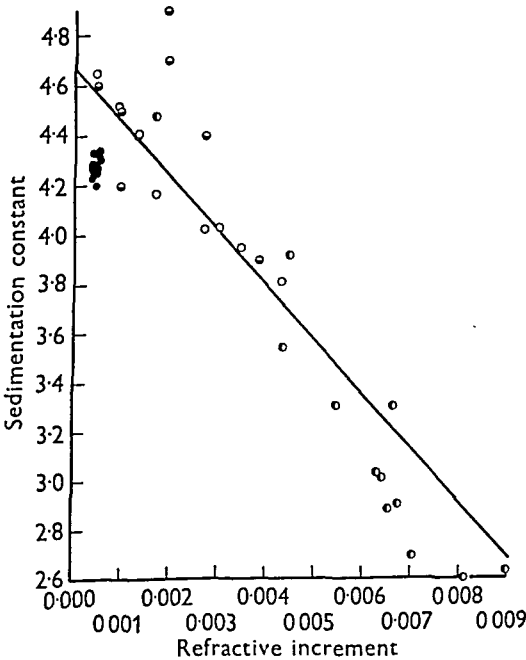


Fig. 3. Values of $s_{20, w}$ for human serum albumin. Points correspond to observations as follows: ○, McFarlane (1935a); ○, Pedersen (1945); ●, Oncley, Scatchard & Brown (1947); ●, this work. The line shown is Pedersen's line of regression.

normal human serum albumin are compared with the values available in the literature. Comparisons must take into account: (a) the different methods of

preparation of the materials, and (b) the reproducibility of the individual techniques of measurement. The figures published by the American workers show a surprising degree of variation, the cause of which is not evident. Although the oil-turbine ultracentrifuge yields a series of sedimentation constants which increase fairly uniformly with decreasing protein concentration, McFarlane's (1935*a*) values are scattered more widely than those of Pedersen (1945). Possible reasons for this are: (a) McFarlane's measurements were made on the albumin peak in serum patterns, not on isolated albumin, and (b) it does not appear that these particular samples were dialysed by him prior to ultracentrifugation. McFarlane (1935*a*) commented that his values did not agree to within the full experimental accuracy, suggesting the presence in serum of a factor which affected the sedimentation constant. This merely expressed the fact that the medium was not sufficiently well defined with respect to its inorganic constituents. The presence of the other proteins in the initial solution was probably not such a great objection since most of the globulins sediment away from the more slowly moving albumin. Although McFarlane's results are generally lower than Pedersen's, one sample of normal human albumin, isolated electrophoretically by McFarlane (1935*b*) and dialysed against 0.1*N*-sodium chloride, gave a sedimentation constant (at 1% concentration) of 4.39, about 2% higher than Pedersen's results would suggest.

At the concentrations used in the Spinco, Pedersen's regression line corresponds to a constant of 4.6. The discrepancy between this and the mean value of 4.28 for the preparations in Table 1 requires careful consideration. It must either be due to errors in the determination or reside in the protein samples themselves. As a check on the latter point a sample of crystalline bovine plasma albumin (Armour), made up to about 0.5% in, and dialysed against, 0.2*N*-sodium chloride solution was used for parallel runs in the Spinco and oil-turbine machines. Mr J. W. Lyttleton at the Lister Institute obtained 4.49 in the oil-turbine, as compared with 4.22 in the Spinco ultracentrifuge.

The factors involved in the determinations have all been considered and possible sources of error checked (see Experimental section). This applies particularly to the temperature, the least satisfactorily defined variable in ultracentrifuge work.

These sedimentation measurements, therefore, whilst individually still subject to errors of about $\pm 2\%$, give a mean value which must be very close to the correct values for this concentration range, and the oil-turbine figures quoted are too high. This is in accord with the conclusions of Cecil & Ogston (1948), who found that, unless the conditions of running of the oil-turbine ultracentrifuge were

carefully standardized, both reproducibility and accuracy could suffer. They decided that in one case (β -lactoglobulin) previous figures were as much as 10% high. A sample of their material (1.2% β -lactoglobulin in a medium consisting of 0.1*N*-sodium chloride, 0.1*N*-sodium acetate, 0.04*N*-acetic acid) was obtained from Oxford and its sedimentation constant measured as additional support for the conclusions formed during this work. The value of 2.78 from the Spinco, compared with 2.80 by the Oxford workers, completely confirmed previous deductions. It is also interesting to note that Johnston & Ogston (1946) obtained sedimentation constants for horse serum albumin which indicated a value of 4.45 at 0.25% concentration. When corrected by the factor 0.978 (Cecil & Ogston, 1948) this becomes 4.35, only slightly different from the Spinco value for human serum albumin.

Diffusion constants

The literature reveals that the diffusion constants of proteins are not known with the accuracy that is desirable. Thus Neurath (1942) commented that, with the Lamm scale method and conventional cells, determinations have standard deviations from the mean of 2–3% for a number of protein preparations.

References to the diffusion of normal human serum albumin are surprisingly few. A determination of the diffusion constant at an unspecified concentration by Longworth & MacInnes (1940) gave a value of 3.34 for $D_{0,w}$, which becomes 6.39 when corrected to 20°. Values of 6.00 (at 0.5% concentration) and 5.85 (at 0.25%) were quoted by Pedersen (1945) for $D_{20,w}$. According to Oncley *et al.* (1947) preliminary measurements have led them to a tentative value of 6.1 for $D_{20,w}$, but no details were given. The values obtained here in the range of concentration 0.2–0.3% have a mean of 6.32, but it has been shown that the differences between the preparations are significant. The experiment at 0.9% ($D_{20,w} = 6.10$) showed that diffusion was not quite Gaussian. Since the process is not highly concentration-dependent this must be attributed to the presence of more than one molecular species in the albumin, a fact already known from electrophoretic work (e.g. Luetscher, 1939; Hoch-Ligeti & Hoch, 1948). Presumably this also accounts for the relatively small differences among the various preparations. Unfortunately, it was not possible to obtain for comparison a sample of human albumin prepared by crystallization in the presence of decanol (Cohn, Hughes & Weare, 1947).

Molecular weights

The mean values for the sedimentation and diffusion constants probably differ by no more than 2% from $s_{20,w}^0$ and $D_{20,w}^0$ respectively. Since both constants increase as the protein concentration

decreases, the error involved in substituting them for $s_{20,w}^0$ and $D_{20,w}^0$ in the calculation of a molecular weight will be even lower. Unfortunately, molecular weights calculated from the combined data of sedimentation and diffusion constants are dependent to a considerable extent on the value of the partial specific volume, \bar{V} , an error of 1% in \bar{V} causing an error of about 3% in the final result. The values of 4.6 for $s_{20,w}^0$ and 6.1 for $D_{20,w}^0$, often quoted (e.g. Oncley *et al.* 1947), give a result of 68 500, but the present work gives 61 500 if we assume the same partial specific volume, 0.733.

SUMMARY

1. The albumin fractions of a number of normal human sera have been isolated electrophoretically.

2. Sedimentation constants for these preparations in the concentration range 0.2–0.3% are con-

siderably lower than the hitherto accepted values. The reasons for this have been thoroughly explored.

3. Diffusion measurements in the Gouy diffusometer show small, statistically significant variations from one preparation to another.

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The Proteins of *Arachis hypogaea* and Fibre Formation

By R. H. K. THOMSON

*Imperial Chemical Industries Ltd. Nobel Division, Fibres Research Department,
 Stevenston, Ayrshire*

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In the course of development of a textile fibre from the proteins of the groundnut (*Arachis hypogaea*) these proteins have recently been the subject of considerable study. Included in this has been the laboratory fractionation of the proteins and an examination of the fractions as fibre-forming agents.

The protein of the groundnut appears to have been first investigated with other vegetable protein

systems by Ritthausen (1880), who extracted the proteins from the oil-free groundnut meal with aqueous sodium chloride and weakly basic solutions, and precipitated them by acidification. Ritthausen considered the solids so obtained to be identical. The investigations of Johns & Jones (1916) have indicated that the total protein of the nut consists of globulins and a very small amount of heat-coagulable albumin. They found it was possible, by

means of ammonium sulphate fractionation of a sodium chloride extract of groundnut meal, to separate the globulins into two fractions—'arachin' and 'conarachin'—which have been found to differ in optical rotation and in the content of sulphur, basic nitrogen, lysine, methionine, cystine, threonine, tryptophan and tyrosine. Later Jones & Horn (1930) stated that arachin could be prepared from 10% sodium chloride extract by dilution until the extract became cloudy, followed by saturation with carbon dioxide, or by the addition of 2 vol. of saturated ammonium sulphate to 3 vol. of the extract. Arachin could also be obtained by dilution alone. Conarachin, which was the more soluble fraction, could not be isolated by dilution, but could be precipitated by dialysis from the filtrate after precipitation of the arachin fraction, or by complete saturation with ammonium sulphate. Conarachin can be coagulated at a lower temperature than arachin. The fractional precipitation of protein mixtures is known to be an arbitrary procedure (cf. Cohn *et al.* 1940) and it is not easy to decide whether the arachin and conarachin are single proteins or mixtures in more or less constant proportions. In order to determine whether these protein fractions were homogeneous, Irving, Fontaine & Warner (1945) conducted electrophoretic analyses on groundnut meal, arachin and conarachin. Their results indicated that the meal contained at least three and probably four components, and that the arachin and conarachin fractions each consisted of mixtures of at least two components. Johnson (1946) investigated the groundnut protein fractions by the ultracentrifuge. He found that the arachin obtained by dilution and addition of carbon dioxide consisted of at least two sedimenting species of protein, while arachin obtained from ammonium sulphate fractionation had only one major constituent. Danielsson (1949) has made a study of seed globulins in the ultracentrifuge and showed the globulins of many leguminous plants, including *A. hypogaea*, to contain 'vicilin' and 'legumin' with molecular weights of 186 000 and 331 000 respectively. He made no attempt to relate his fractions to arachin and conarachin. Karon, Adams & Altschul (1950) have shown by electrophoretic measurements that groundnut protein consists of two major and several minor components. If the protein was separated from a meal which had been washed with water and adjusted to pH 5 to remove soluble sugar and phytin, the major component separated into two almost equal fractions. There is evidence of the close relationship between the protein components of the groundnut in the interconversion from one component to the other which takes place in alkaline buffer solutions.

Johnson, Joubert & Shooter (1950), in more recent work, have investigated reversible dissociation of the arachin fraction. They have shown that the

parent molecule of this fraction with a molecular weight of about 400 000 dissociates under appropriate conditions into two sub-units of molecular weight 200 000. The parent and sub-molecules are very easily distinguishable with the ultracentrifuge.

From the literature it appeared that the most practical method for preparing protein fractions in experimental quantities was fractionation by differential solubilities. Differential solubility in various concentrations of sodium chloride, indicated by Jones and his co-workers and Johnson and his co-workers, seemed the simplest procedure. The possibility of fractionation using salts other than sodium chloride and even aqueous solvents other than salt solutions has not been overlooked, but the work reported here has been restricted to the use of sodium chloride. Methods of fractionation based on electrophoresis or ultracentrifugation appeared to offer no opportunities on the scale necessary.

EXPERIMENTAL

In the literature the principal method of extraction of the protein from groundnut meal is by extraction with salt solution. In the extraction of protein for fibre manufacture by the 'Ardil' process described by Traill (1945) dilute aqueous caustic soda (0.1 g. NaOH/l. of water) is employed, and the protein is precipitated from this solution by adjusting to the pH of lowest solubility, which is 5. Protein extracted and precipitated in this way may be washed and spray-dried. This spray-dried product is the starting point for the investigation described in the present paper.

The work on fractionation of spray-dried groundnut protein divided itself into three main sections:

(a) The investigation of the solubility of spray-dried protein from nuts from various sources in aqueous solutions of NaCl.

(b) The study of the fractions present by a chromatographic technique.

(c) Isolation of suitable fractions in sufficient quantity for tests of fibre-forming properties.

The investigation of the solubility of spray-dried groundnut protein from nuts from various sources in aqueous solutions of sodium chloride

The separation of groundnut protein into two fractions, one of which was soluble in both 10 and 2% (w/v) aqueous NaCl in slightly acid solution, and one which was soluble in 10%, but insoluble in 2% aqueous NaCl under the same conditions, may be a considerable simplification of complex solubility phenomena. It was decided to study in more detail the effects of varying salt concentration and pH value over a limited range. This range was from 0 to 10% NaCl concentration and pH 1–7, and within this range the protein solubilities were determined. Spray-dried protein (4 g.) was shaken in 100 ml. aqueous salt solution of the stated concentration, the pH was adjusted with NaOH or HCl (dilution due to this was kept negligibly small) until equilibrium was attained at the required value and the mixture was filtered. The residue was washed with large volumes of methylated spirit to remove the water and at least some of the salts

and then the methylated spirit was washed out with acetone. The residue was oven-dried and weighed. By this method the protein which remains behind on the filter paper will be contaminated with NaCl. The errors caused by this salt were less than 2% of the weight of residual protein. Difficulties were encountered because in each pH range at certain salt concentrations the whole of the protein residue swelled and became jelly-like. Under these conditions the

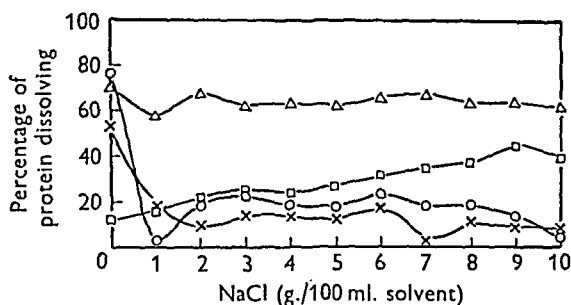


Fig. 1. Fractional solubility of groundnut protein (batch 1) in aqueous sodium chloride. For details see text. Δ , pH 7; \square , pH 5; \times , pH 3; \circ , pH 1.

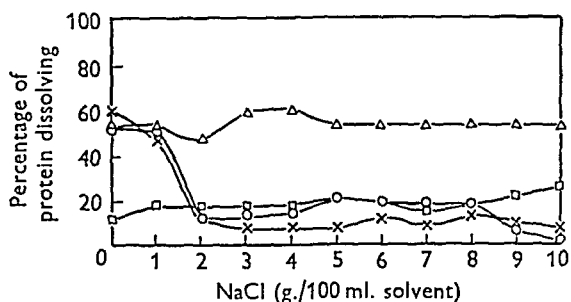


Fig. 2. Fractional solubility of groundnut protein (batch 2) in aqueous sodium chloride. For details see text. Δ , pH 7; \square , pH 5; \times , pH 3; \circ , pH 1.

distinction between soluble and insoluble was far from clear-cut. Figs. 1 and 2 show the solubilities of protein from two samples of groundnuts (from two different areas in Africa) in aqueous NaCl solutions (0–10%, w/v) in the pH range 1–7. Such data are best considered as a solid diagram, and such solids can be visualized from Figs. 1 and 2, the curves being cross-sections. In Fig. 1 the following may be observed: (i) the point of minimum solubility (pH 5 in water) becomes more acid as salt concentration increases; (ii) at pH 7 the solubility is high at all salt concentrations; (iii) there is a fall in solubility between salt concentrations of 9 and 6% at pH 5. This last appears to be due to the insolubility of Jones's arachin at low salt concentration. In Fig. 2 it may be seen that the fall in solubility described in (iii) is not present. The protein of Fig. 2 appears to be deficient in arachin. It may be seen that separation into the two solubility forms of Jones (both soluble in 10% salt but only one soluble in 2% salt) can be carried out at pH 5. This pH value corresponds reasonably with that attained by Jones on the addition of CO_2 and exactly with the pH value used by Johnson. The spray-dried protein from both sources contains a fraction which is insoluble in 10% salt; this at pH 7 amounts to about 30% of the whole protein and at pH 5 to about 50%.

The study of fractions present by a chromatographic technique

At this stage in the work the need for a rapid method for identifying arachin and conarachin was felt. A chromatographic technique was evolved which showed considerable promise.

If a 10% (w/v) aqueous solution of NaCl is allowed to diffuse from a reservoir across a filter paper already wet by water, the diffusion front will not be sharp but will consist of a boundary area with salt concentrations varying from 0 to 10%. The movement of this boundary area will be accelerated by permitting free evaporation of water from the filter

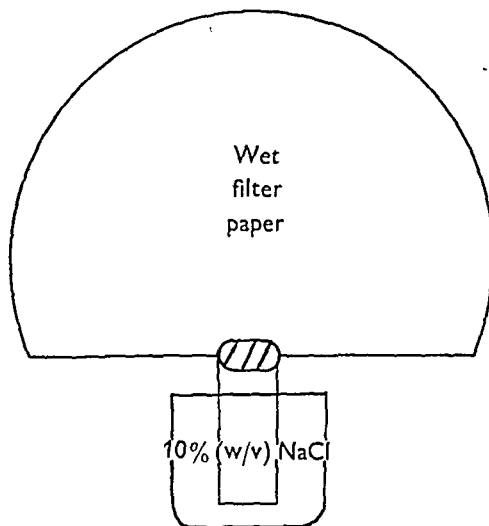


Fig. 3. Method of making protein chromatogram. Protein solution is applied over hatched area.

paper. It has been found that if the first of the diffusing salt solution also had groundnut protein in solution, separation into two forms, arachin and conarachin, occurred at the diffusion front. This was most easily observed by dyeing the protein on the paper by the method of Jones & Michael (1950). A study has not yet been made of the mechanism of this separation, but it seems likely that displacement chromatography or salting-in chromatography may occur (Shepard & Tiselius, 1949; Swingle & Tiselius, 1951; Tiselius, 1948). The practical details of the development of the chromatogram are illustrated by reference to Fig. 3. A filter paper is cut into the mushroom shape shown and is soaked with distilled water. Any surplus is removed by blotting. One drop of a solution of groundnut protein in 10% aqueous NaCl is placed in the area shaded. This forms a barrier above the wick which dips into a vessel containing 10% aqueous NaCl. As the water from the paper evaporates the salt solution spreads upwards and outwards. After about 15 min. the paper is ready for dyeing.

Using this method the arachin and conarachin separated quite clearly into lines with a separation of 2–3 mm. (Fig. 4). The front line, being in the more dilute part of the zone, was identified as conarachin and the rear line as arachin. The identity of these lines was tested in several ways. According to Jones & Horn (1930) conarachin is precipitated by heating. A solution of the protein in 10% NaCl solution which gave two lines originally, gave only one

after boiling and filtering off the precipitate (Fig. 5). This one line was presumably the arachin line. If arachin is precipitated from the original 'two-line' solution by dilution and filtered off, the filtrate should consist of conarachin alone. When such a filtrate was fortified with salt to 10% again the resulting figure showed one line only, the conarachin line. This solution, mixed with the arachin filtrate from the previous experiment, gave a two-line diagram.

arachin. The residue was extracted with 10% (w/v) aqueous NaCl to give a solution of arachin and a residue of the fraction insoluble in 10% (w/v) NaCl. Alternatively, whole protein was extracted first with 10% (w/v) aqueous NaCl to give a solution of arachin and conarachin, and a residue insoluble in 10% (w/v) NaCl. The solution was diluted with four times its volume of water to precipitate the arachin. Conarachin remained in solution.

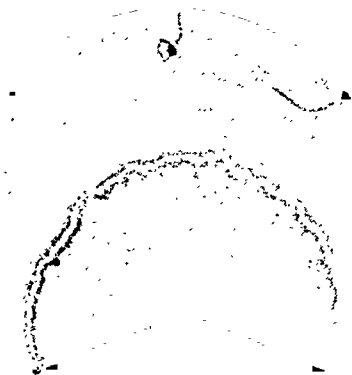


Fig. 4. Chromatogram of groundnut protein in aqueous sodium chloride. Conarachin line outside arachin line.

Similar confirmation was obtained by using the dilution precipitate as the source of arachin and the 2% salt extract of the whole protein as the source of conarachin.

Two routes are available for the preparation of arachin and conarachin from spray-dried groundnut protein at pH 5.

In a study of these routes whole protein was extracted first with 2% (w/v) aqueous NaCl to give a solution of con-

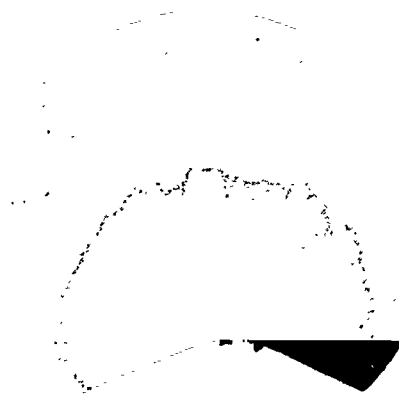


Fig. 5. Chromatogram of groundnut protein boiled in aqueous sodium chloride. Arachin line showing.

Fig. 6 shows a full investigation of the two routes using the chromatographic technique. From this there is no evidence of a form insoluble in 10% (w/v) aqueous NaCl and soluble in 2% (w/v) NaCl.

It would also appear that the chromatographic technique separates the proteins into the same fractions as solubility

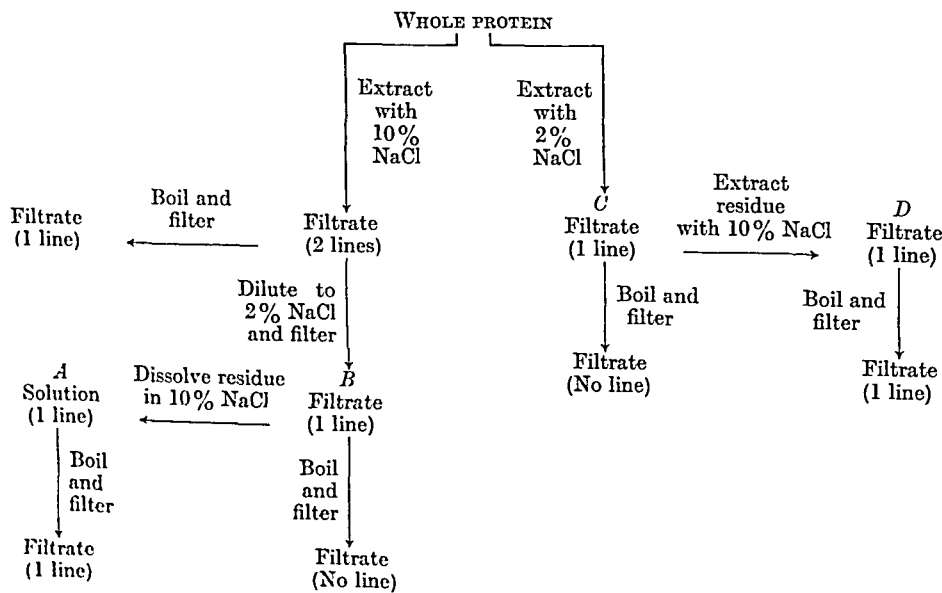


Fig. 6. Chromatographic examination of fractions of groundnut protein.

Table 1. *Fibre-forming properties of fractionated groundnut protein*

(A = arachin; C = conarachin; X = insoluble in 10 % aqueous NaCl at pH 5.)

Fraction	Constituents	Extruded solution (g./100 g. water)		Fibre-forming properties	Remarks
		NaOH	Protein		
Whole protein	ACX	1.1	26	Good	Discrete filaments, easily stretched
Insoluble in 10 % (w/v) NaCl	X	0.8	19	Good	
Insoluble in 2 % (w/v) NaCl	AX	1.0	21	Good	
Arachin	A	0.9	24	Good	
Soluble in 10 % (w/v) NaCl	AC	0.7	18	Bad	Stuck filaments, difficult to stretch
Conarachin	C	0.8	15	Unspinnable	

Filaments, when formed, too weak to remove from coagulating bath

methods and shows no sign of the complexities in the solubility fractions shown by ultracentrifuge and electrophoresis work. No examination of the present preparations was made by the latter two methods, and, until this is done, it must be supposed that the chromatographic separation is as arbitrary as Cohn *et al.* (1940) consider fractional precipitation to be.

Isolation of suitable fractions for fibre-formation tests

The primary object of this research is the development of a protein fibre made from groundnut protein. Experience has shown that variations occur in the fibre-forming properties of different lots of protein. An accurate method of measurement of these fibre-forming properties has yet to be developed, but in filament extrusion in the laboratory, in accordance with the 'Ardil' process, there is no difficulty in assessing fibre-forming properties as good, bad or unspinnable. This assessment is based on the ability, on extrusion from a many-holed spinneret, to form discrete filaments which, during coagulation, may be stretched to several times the extruded length and may be wound out of the coagulating bath. Where these conditions are fulfilled, fibre formation may be regarded as good. Where the first two conditions are not fulfilled, but the third is, the fibre formation is bad. Where all three are unfulfilled the material is unspinnable.

With this as a basis, extrusion experiments were performed on whole groundnut protein and on suitable fractions of the same lot of protein. The protein fractions were prepared for extrusion by dissolving them in dilute aqueous caustic soda in accordance with the directions of Traill (1945). After maturing for 20 hr. at 20° solutions of suitable viscosity were obtained. These solutions were extruded through viscose spinnerets into a coagulating bath at 30° composed of 11 ml. H₂SO₄ and 250 g. Na₂SO₄ dissolved in 1 l. of water. Table 1 shows the fractions used, the composition of the solution extruded and the results obtained.

The mixed fraction which is insoluble in 2 % (w/v) NaCl (Table 1) contained arachin and insoluble fractions in the same proportions as they occurred in the parent protein, and similarly for the arachin and conarachin in the mixed fraction which was soluble in 10 % (w/v) sodium chloride. Both arachin and insoluble fractions were good fibre-formers and conarachin was not. A mixture of arachin and the insoluble fraction was also a good fibre-former. Conarachin could be tolerated in the proportion in which it

occurs in the whole protein, but when the proportion was increased by removing the insoluble fraction, fibre formation deteriorated.

No made-up mixtures of the fractions were studied in these experiments, but it is proposed to continue the work with such studies.

DISCUSSION

The protein of the groundnut, whether extracted by aqueous sodium chloride or by aqueous caustic soda is of some complexity. The position is not greatly clarified by considering the differences in the components shown by the solubility evidence, the ultracentrifugal evidence and the electrophoretic evidence. From the point of view of commercial fibre formation the presence of a considerable fraction showing no fibre-forming properties whatever is interesting. There is immediate need for a study of the interaction of three selected fractions (arachin, conarachin and insoluble fraction) on the properties of the finished fibre. The fraction which is insoluble in 10 % (w/v) aqueous sodium chloride at pH 5 requires further study. Some of this fraction is soluble in 10 % sodium chloride at pH 7 while more of it is not. It may well consist of a less soluble derivative of either arachin or conarachin, but no experimental evidence is presented in support of this view.

SUMMARY

1. The solubility has been determined of groundnut protein in aqueous solutions of sodium chloride at concentrations up to 10 % (w/v) and over the pH range 1-7.

2. A paper-chromatographic technique has been developed which demonstrates the presence or absence of arachin and conarachin.

3. Arachin, conarachin and a third fraction which is insoluble in 10 % aqueous sodium chloride have been studied as fibre-forming agents.

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A Technique for the Identification and Separation of Enzymes by Paper Chromatography

By K. V. GIRI, A. L. N. PRASAD, S. GOWRI DEVI AND J. SRI RAM
Department of Biochemistry, Indian Institute of Science, Bangalore

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Paper partition chromatography, first described by Consden, Gordon & Martin (1944), has proved an effective method for separating the simpler chemical compounds from complex mixtures such as protein hydrolysates and for identifying them on a micro-scale. So far, paper partition chromatography has been used chiefly in the analysis of amino-acids, carbohydrates, purines, nucleic acids, organic acids, vitamins and other substances of biological importance. Very little is recorded in the literature on the application of this technique or of adsorption chromatography on paper to the study of enzymes, except the recently published reports by Franklin & Quastel (1949), Mitchell, Gordon & Haskins (1949) and Reid (1950). Franklin & Quastel (1949) have reported their preliminary investigations on the enzyme urease. They studied the movement of this enzyme on paper, using a cysteine-glycine solution as the developing solvent and determined the activity of the enzyme on paper, manometrically. They showed that the movement of the enzyme on paper can be followed by this technique, without the enzyme losing its activity during the experiment. However, they did not attempt to separate enzymes by this technique. Mitchell *et al.* (1949) made use of the 'chromatopile' (a pile of filter-paper disks) for the separation of the constituents of takadiastase preparations, by placing the enzyme mixture near the top of the filter-paper pile and fractionating by a process involving solubility in a concentration gradient. They report that some separation of adenosine deaminase from amylase was obtained by using aqueous ammonium sulphate as the solvent. In these methods the enzymes are located by carrying out a series of determinations of the activity of the enzymes

present in different parts of the paper. This procedure is very cumbersome and time-consuming for carrying out preliminary investigations on the movement of enzymes on paper. As a consequence, we have employed a simpler technique for locating the enzymes on paper, using agar plate containing the substrate on which the enzyme acts and suitable reagents for the detection of the hydrolysed products (cf. Goodall & Levi, 1947). The ease with which the enzymes on paper can be located by means of this technique prompted us to investigate the movement of enzymes on paper, with a view to separating enzymes by paper chromatography. Reid (1950) has given a preliminary account of similar studies, chiefly with fungal enzymes. The essential feature is the use of precipitating solvents such as aqueous acetone or alcohol or salt solutions as the moving phase (for references see Swingle & Tiselius, 1951). A preliminary account of this technique has already been given (Giri & Prasad, 1951).

It is intended in the present paper to demonstrate the usefulness and potentialities of this technique in the study of the chromatographic behaviour of enzymes and to describe some examples of the separation of enzymes, which have so far been achieved in preliminary investigations of some important enzyme systems. The study of other enzymes is in progress.

MATERIAL

The various enzyme preparations used in the present investigations were either isolated as dry powder by precipitation or obtained as aqueous extracts from natural sources.

Amylases

Amylase from sweet potato. This enzyme was prepared from aqueous extract of the dried powder of sweet potatoes by precipitation with 4 vol. of 95 % (w/v) aqueous ethanol as described by Giri (1934). 0.10 g. of the preparation was dissolved in 1 ml. of water and centrifuged to remove the suspended impurities, and the clear aqueous extract was used.

Amylase from saliva. The enzyme was prepared from saliva according to the method of Hanes & Cattle (1938). 0.30 g. of the enzyme preparation was dissolved in 10 ml. of water.

Amylase from Aspergillus niger. The amylase solution was prepared by extraction of 1 part of dry mycelium with 10 parts of water for 4 hr. and subsequent filtration. The clear filtrate was used, without further treatment, as source of the enzyme.

Amylase from rice. Germinated and dried rice powder was extracted with 8 times its volume of water and the enzyme mixture was precipitated from the extract by the addition of three volumes of acetone. The precipitate obtained from 15 ml. of original extract was dissolved in 0.25 ml. of water.

Phosphatases

Kidney and liver phosphatases. The enzyme preparations were obtained from acetone powders which were prepared from the kidney and liver (sheep and rat) in the conventional manner. The tissues were washed free from blood with cold water and minced well. The minced tissue was ground with acetone and filtered. It was treated with a further quantity of acetone followed by ether and filtered. The preparation thus obtained was first dried in a current of air and then in a vacuum desiccator over H_2SO_4 . The dry material was ground finely and used for extraction of the enzymes.

For extraction, 1.0 g. of the powder was triturated with 20 ml. of cold water for 2–3 hr. The insoluble material was removed by centrifugation. The supernatant liquid was kept in a refrigerator under toluene. This solution was used directly.

Serum phosphatase. Human, rat and chick sera were used as such as source of the enzyme.

Phosphorylase

Green gram (*Phaseolus radiatus*), both resting and germinated, was used as source of the enzyme. The resting seeds were powdered, sieved (100 mesh) and extracted with 5 vol. of water for 3–4 hr. at 0° . The clear solution obtained on centrifugation was used as a source of the enzyme. For the preparation of enzyme extract from the germinated seeds, the same proportion of water to the weight of the seeds was used for extraction.

METHODS

With a micropipette (7–12 μ l.) known volumes of the enzyme solutions were placed on a filter paper (Whatman no. 1; 45–50 cm. long and 14–18 cm. wide) at intervals of about 2.5 cm. on a line drawn about 10 cm. from one end of the filter paper. Care was taken to confine the enzyme to a spot of as small an area as possible (about 1 cm. in diameter). A total of 50 μ l. of the enzyme solution could be deposited on the paper within such an area by applying it in 7–10 μ l. portions and allowing to dry before the next application. After drying, the paper was hung from a glass trough fitted near the top of a rectangular glass chamber. The whole assembly was kept in a refrigerator at $0-5^\circ$.

The bottom of the glass chamber was covered with the aqueous solution to maintain suitable conditions of humidity. The solvent was then poured into the glass trough and allowed to spread down the paper sufficiently far beyond the starting line. Usually the chromatogram was allowed to run until the solvent had advanced about 20 cm. from the starting line. Approximately 4–8 hr. run was sufficient in most cases. With *n*-butanol as solvent, however, about 16–20 hr. were necessary for the solvent front to travel the same distance as the other solvents. After the solvent had travelled a convenient distance, the paper strip was removed and the limit of excursion of the solvent marked. The paper was allowed to dry at room temperature. The position of the enzymes on the paper was located as follows.

Identification of enzymes on the paper

The agar-substrate media used for the detection of the various enzymes consisted of 2 g. agar-agar, with the following additions, made to final volume 100 ml. with water.

Amylases. 'Soluble starch,' 1 g. (British Drug Houses Ltd.) and 30 ml. of 0.2M-sodium acetate buffer (pH 4.6) for sweet-potato amylase or 30 ml. of 0.067M-phosphate buffer ($KH_2PO_4 + Na_2HPO_4$; pH 7.0) for salivary and *Aspergillus niger* amylases.

Phosphatases. Sodium phenolphthalein phosphate (0.1 g.) and 30 ml. of 0.02M-sodium acetate buffer (pH 5.2) for acid phosphatase or 30 ml. of 0.1M-sodium glycine buffer (pH 9.2) containing also 0.58 % (w/v) NaCl for alkaline phosphatase.

Phosphorylases. Glucose-1-phosphate, 0.2 g. (K_2 salt) and 30 ml. of 0.2M-sodium citrate buffer (pH 6.0).

The mixtures were usually made after autoclaving the agar solution. The agar-substrate medium, while still hot, was poured on a glass plate 16 by 8 in. This was allowed to cool. The dried paper-strip chromatogram was laid gently on the agar plate and allowed to remain on it for 4–12 hr. at room temp. ($20-30^\circ$), depending on the activity of the enzyme. The paper was then removed gently without disturbing the surface of the agar layer and the surface was flooded with the appropriate reagent. Iodine solution (0.01 N) was used for the detection of amylases and phosphorylases and 0.1 N-NaOH for the detection of phosphatases. We could also locate the position of phosphatases by locating the inorganic P formed by their action on sodium glycerophosphate. It was, however, found that clear spots were obtained using phenolphthalein-phosphate as substrate. The positions of the enzymes on paper were clearly indicated by the formation of coloured or colourless spots on the agar plate. The presence of β -amylase or α -amylase was indicated by the formation of violet or colourless spots respectively against a blue background. Phosphorylase produced a blue spot while phosphatases produced pink spots against a colourless background. The position of the enzymes on the paper was also indicated by spraying the above reagents on the paper itself. It was, however, found that well-defined and clear spots were best obtained on the surface of the agar plate.

For locating on the same chromatogram enzymes which hydrolyse the same substrate at different pH's (such as the alkaline and acid phosphatases) or act on different substrates (like phosphorylase and amylase) the paper was cut into strips longitudinally, each carrying the enzyme concerned. The strips were then placed on the agar plate containing suitable substrate and adjusted to suitable pH.

Developing solutions

The choice of developing solution for the chromatography of enzymes is rather limited on account of the labile nature of enzymes. It appeared probable that the movement of enzymes on paper might be obtained by using the ordinary solvents employed for precipitating enzymes. Aqueous acetone, aqueous ethanol and saline solution were, therefore, chosen for trial. *n*-Butanol saturated with water was also tried, as it was found to have no deleterious effect on the enzymes. After a series of trials with all these solvents mixed in various proportions with water, it was found that aqueous acetone (20–50%, v/v) and sodium chloride (2–20%, w/v) proved the most useful developing agents for the separation of some of the enzymes investigated. The enzymes did not travel at all when *n*-butanol saturated with water was used as solvent.

RESULTS

Movement of enzymes in various developing solutions

The technique described was first applied to the study of the movement of various important enzymes and later extended to the resolution of the individual enzymes in mixtures and in extracts of plant and animal tissues. Table 1 gives the position on the chromatograms of the enzymes from plant and animal tissues and blood sera in various developing solutions. The R_F values (Consden *et al.* 1944) represent the average of several experiments. This table is intended to serve as a guide to the relative positions of the enzymes on paper, under the experimental conditions. The movement of the enzymes is influenced by the nature of the accompanying substances present in the enzyme preparations employed, the concentration of the enzyme, the nature of the developing solution and other unknown factors.

It will be apparent from the data given that some of the enzymes investigated have high R_F values while others do

not show any movement at all. Aqueous ethanol (50%, v/v), acetone (50%, v/v) and NaCl solution (2%, w/v) bring about the movement of many of the enzymes investigated. β -Amylase (from sweet potato), amylase from *A. niger*, phosphorylase from green gram and amylase from germinated rice show considerable movement, while α -amylase (salivary) does not move at all. One of the alkaline phosphatases of kidney and the alkaline phosphatases of rat and human sera also do not show any movement.

Solvents such as *n*-butanol and acetone containing very little water do not bring about the movement of the enzymes.

Chromatography of mixtures of enzymes

The observation that enzymes move on paper without losing their activity and that the rate of movement differs from one enzyme to another depending on their degree of aggregation and the strength of association with other proteins and accompanying substances indicates the basis for a chromatographic separation of enzymes. The technique described above may, in fact, be applied to the separation of enzymes from one another, and examples of the separation of enzymes which has so far been achieved in preliminary investigations are given below.

Separation of α -amylase (salivary) and amylase from A. niger. We have succeeded in separating the components of such a mixture, using aqueous acetone (50%, v/v) as the developing solvent. A typical chromatogram of a mixture of these two amylases is shown in Fig. 1. It can be seen from the figure that the separation of the two amylases is readily achieved. The salivary amylase remains at the starting point, while the amylase of *A. niger* travels down the paper. Thus it is possible by means of this technique to separate from one another even closely related enzymes like the two α -amylases. Although a mixture of salivary amylase and the amylase of *A. niger* can be separated, the complicated shape of the chromatogram obtained in the case of a mixture of salivary amylase and β -amylase

Table 1. *Behaviour of enzymes on paper chromatograms*

Enzyme	Source	Solvent used	R_F
α -Amylase	<i>Aspergillus niger</i>	Aqueous acetone (50%, v/v)	0.75
β -Amylase	Sweet potato	Aqueous acetone (50%, v/v)	0.50
α -Amylase	Saliva	Aqueous acetone (50%, v/v)	0.00
Amylases	Germinated rice	0.33 M-NaCl	0, 0.67*
Amylases	Germinated rice	Acetone-water 75/25 (v/v)	0
Phosphorylase	Green gram (14 μ l.)	0.33 M-NaCl	0.77
	(21 μ l.)	0.33 M-NaCl	0.74
	(28 μ l.)	0.33 M-NaCl	0.65
	(42 μ l.)	0.33 M-NaCl	0.55
	(28 μ l.)	3 M-NaCl	0.68
Acid phosphatase	Rat kidney	0.33 M-NaCl	0.61
Alkaline phosphatase	Rat kidney	0.33 M-NaCl	0.00, 0.67*
Acid phosphatase	Sheep kidney	0.33 M-NaCl	0.53
Alkaline phosphatase	Sheep kidney	0.33 M-NaCl	0.00, 0.61*
Acid phosphatase	Sheep kidney	Aqueous acetone (30%, v/v)	0.53
Alkaline phosphatase	Sheep kidney	Aqueous acetone (30%, v/v)	0.00, 0.60*
Acid phosphatase	Rat liver	0.33 M-NaCl	0.63
Alkaline phosphatase	Rat liver	0.33 M-NaCl	0.67
Alkaline phosphatase	Chick serum (22 μ l.)	0.33 M-NaCl	0.65
Alkaline phosphatase	Human serum (88 μ l.)	0.33 M-NaCl	0.00
Alkaline phosphatase	Rat serum (55 μ l.)	0.33 M-NaCl	0.00

* Two components.

from sweet potato renders the separation of these two enzymes from one another somewhat difficult at the present stage.

Amylases from germinated rice. Similarly, clear separation of amylases occurring in aqueous extracts of germinated rice into two enzymes, one having no movement at all and the other having an R_F value of 0.67 was achieved using NaCl solution (Fig. 2). We have not as yet been able to characterize these two amylases.

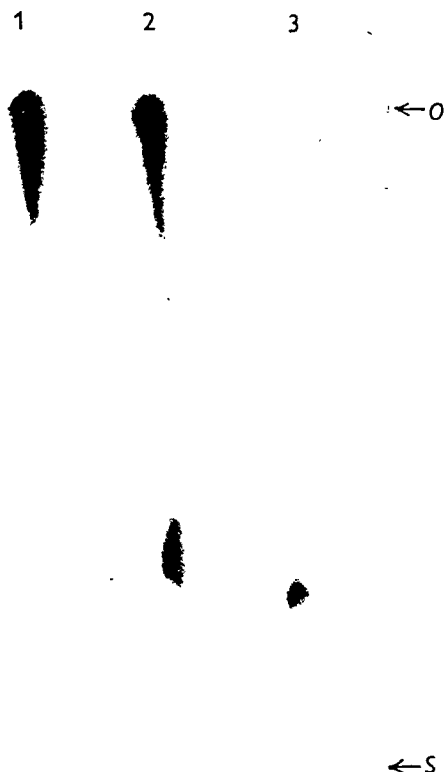


Fig. 1. Chromatograms of a mixture of α -amylase (salivary) and amylase from *A. niger* 50% (v/v) aqueous acetone: O, starting line; S, solvent front; 1, α -amylase (salivary); 2, α -amylase (spot at the starting line) and amylase from *A. niger* (spot at the bottom); 3, amylase from *A. niger*.

Kidney phosphatases. Clear separation of the phosphatases present in rat, guinea pig and sheep-kidney extracts into two distinct alkaline phosphatases (one of them having an R_F value of 0.62–0.68 and the other showing no movement) and one acid phosphatase with an R_F value of 0.53–0.61 which moves slightly more slowly than the corresponding alkaline phosphatase was achieved by this method (Fig. 3). Further investigation is in progress on the characterization of the two alkaline phosphatases which have been chromatographically shown to be quite distinct from one another. Pending further knowledge of the nature of these phosphatases it is proposed to name them as stationary and mobile phosphatases.

Liver phosphatases. The examination of the chromatograms (Fig. 3) reveals the presence of mobile acid and alkaline phosphatases.

Serum phosphatases. The chromatograms of the phosphatases of human and rat sera showed the existence of alkaline and acid phosphatases both of which were stationary, while

the chromatogram of chick serum phosphatase showed the existence of a mobile phosphatase. The difference in the movement of the phosphatases of rat and human sera on the one hand and that of chick serum on the other is rather striking. This characteristic difference in the chromato-

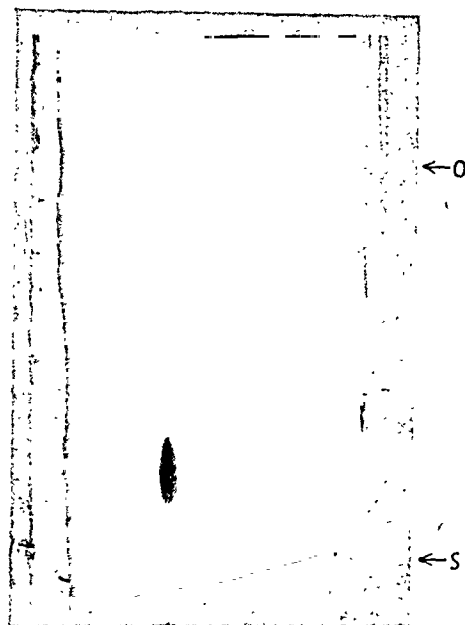


Fig. 2. Chromatogram of amylases of aqueous extract of germinated rice showing the presence of two distinct amylases. One remains at the origin while the other moves down. 2% (w/v) aqueous NaCl; O, starting line; S, solvent front.

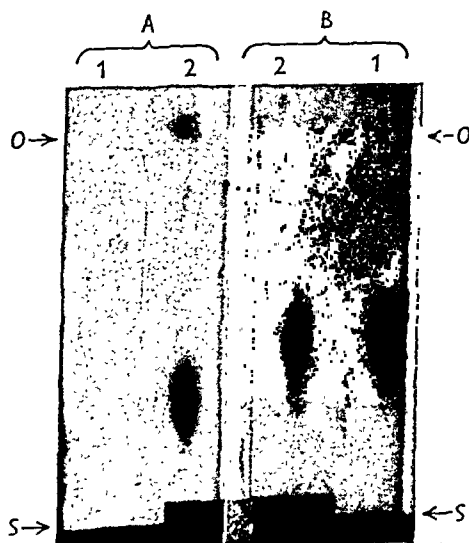


Fig. 3. Chromatograms of phosphatases from kidney and liver of rat. 2% (w/v) aqueous NaCl; O, starting line; S, solvent front; A1, alkaline phosphatase of liver; A2, alkaline phosphatase of kidney; B1, acid phosphatase of liver; B2, acid phosphatase of kidney.

graphic behaviours of the sera of various animals in both normal and pathological conditions is being investigated.

Amylase and phosphorylase of green gram. Our attempts to separate the amylase and phosphorylase of germinated green gram using various solvents did not prove fruitful due to the fact that both enzymes moved together. Fig. 4 shows the interesting fact that on developing the agar plate with iodine solution there is a small break in the middle

lase, alkaline phosphatase of human serum and one of the alkaline phosphatases of kidney do not show any movement at all, and they are found at the starting point, irrespective of the solvent used. The other enzymes investigated, namely, amylase from *A. niger*, β -amylase from sweet potato, phosphorylase from green gram, the acid phosphatase and one

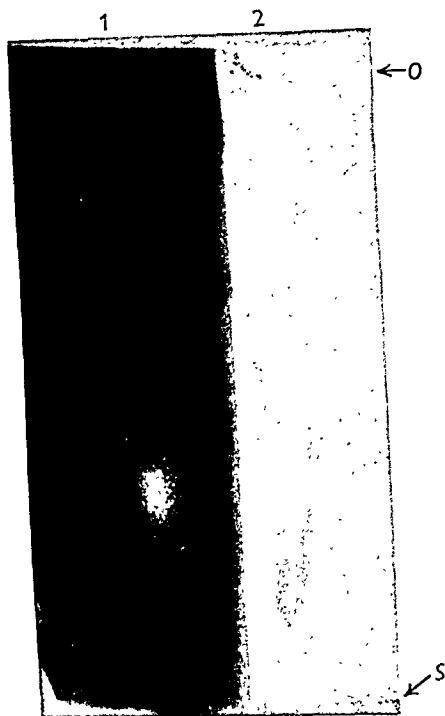


Fig. 4. Chromatograms of the amylase and phosphorylase of aqueous extract of germinated green gram (*Phaseolus radiatus*). 2 % (w/v) aqueous NaCl; O, starting line; S, solvent front. 1, amylase; 2, phosphorylase (showing the position of the amylase by the absence of phosphorylase activity at the centre of the spot).

of the long blue spot. This is clearly the position of the amylase which either prevents the phosphorylase from acting on the Cori ester present in the agar or hydrolyses the starch synthesized from it.

DISCUSSION

The present data offer an idea of the chromatographic behaviour of enzymes on paper. Most of the enzymes investigated travel on paper in solvents with high water content, such as aqueous acetone or alcohol and salt solutions. From the above results of preliminary investigations certain generalizations on the movement of enzymes on paper may be made. (1) Enzymes can be made to move on paper by selecting suitable solvents. The rates of movement of the enzymes investigated differ from one another. Some enzymes such as salivary amy-



Fig. 5. Chromatograms of phosphorylase from green gram (*Phaseolus radiatus*) showing the influence of concentration of the enzyme on the rate of movement. 2 % (w/v) aqueous NaCl; O, starting line; S, solvent front. 1, 14 μ l.; 2, 28 μ l.; 3, 42 μ l. enzyme solution applied to paper.

of the alkaline phosphatases of kidney, acid phosphatase of liver and one of the amylases of germinated rice grain show considerable movement. (2) Increase in the concentration of the enzyme decreases its rate of movement. This has been observed particularly in the case of phosphorylase with 2 % (w/v) sodium chloride as the solvent (Fig. 5). (3) The R_f values of the enzymes are also influenced by the distance to which the solvent front has advanced. This is clearly shown in the case of the amylase from *A. niger* with aqueous acetone as solvent. The R_f values decrease slightly with increase in distance travelled by the solvent front (Giri & Prasad, 1951). (4) The rate of movement of the enzymes is slower with aqueous acetone than with sodium chloride as solvent. (5) Proteins and other non-enzymic impurities associated with them

tend to alter the movement of the enzyme or suppress it altogether. Separation of enzymes under such conditions can still be achieved by choosing a suitable solvent. It may be that in some instances the lack of movement of enzymes on paper is due to protein interaction with other impurities present in the extract or adsorption of the enzyme on to other substances which are absorbed on paper. Too little is known about these factors. For this reason it is clearly important that the material used should be as free as possible from impurities, and exact conditions which give complete separation must be determined for each of the investigated enzymes. (6) The observation that there are two distinct and well-defined spots in the chromatogram of the alkaline phosphatase (Fig. 3) of the acetone-treated kidney extract would indicate the existence of at least two distinct alkaline phosphatases in kidney. The chemical basis for the differences between the alkaline phosphatase is not understood. In crude extracts the presence of substances acting as adsorbents or protective agents might contribute to the observed differences in the movement of the two enzymes. However, whatever effects such substances may have, the two phosphatases retain their individuality irrespective of the solvent used. But this evidence should be confirmed by the isolation of the enzymes by chromatography using cellulose columns, and study of their characteristics before their non-identity is finally accepted. The difference in the mobility of the enzymes may lie in the enzymes themselves, or be due to something which remains firmly associated with the enzyme. In addition to the existing numerous histochemical and biochemical methods for distinguishing the phosphatases from one another, the paper-chromatographic method may provide a further basis for distinguishing these enzymes present in various tissues. (7) The results are easily reproducible as

long as the same experimental conditions are maintained. When duplicates are run simultaneously on the same paper, the differences in R_F values are negligible. Under the experimental conditions it was found possible to reproduce with reasonable exactness the characteristic positions occupied by the enzymes.

The paper-chromatographic method is undoubtedly a useful addition to the methods used in enzyme chemistry for determining the identity or diversity of enzymes and for separating individual enzymes from a mixture. It is obvious that the chromatographic behaviour of enzymes may provide a basis for the differentiation of enzymes of overlapping or identical substrate specificity. The very small amounts of the enzyme preparations used in these methods make preparative work difficult. For preparative work based on the same principle columns made from powdered paper may, however, prove satisfactory.

SUMMARY

1. A technique for the identification and separation of enzymes by paper chromatography is described.
2. The application of this technique to the study of the movement of amylases, phosphorylases and phosphatases on paper has been examined and R_F values tabulated.
3. Aqueous acetone, aqueous ethanol and sodium chloride solutions have proved useful as solvents for the study of the movement of the enzymes on paper.
4. Some examples of the separation of enzymes by means of this technique are given.
5. The usefulness and potentialities of this technique in the study of the chromatographic behaviour of enzymes are discussed.

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Dextran Sulphate—A Synthetic Analogue of Heparin

By C. R. RICKETTS

*Medical Research Council Burns Unit, Birmingham Accident Hospital,
Bath Row, Birmingham 15*

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The structure of the natural anticoagulant, heparin, is gradually being elucidated. As each step forward is made, attempts to synthesize molecules with the biological activity of heparin receive renewed impetus. It is now known (Jorpes, 1939) that heparin is composed of glucuronic acid and glucosamine though the mode of linkage of these two parts is not yet clear. Some of the hydroxyl groups of these components are esterified with sulphuric acid but the exact distribution of sulphate groups is uncertain. Recently, however, Jorpes, Bostrom & Mutt (1950) have shown that during the weak acid hydrolysis of heparin, sulphuric acid appears at the same time and with the same rate as amino groups, leading to the view that the amino group is combined with sulphuric acid. This conclusion is confirmed by the work of Meyer & Schwartz (1950).

An important contribution to our knowledge of the size of heparin molecules was made by Grönwall, Ingelman & Mosiman (1945), who measured sedimentation and diffusion constants for a substantially pure preparation in the ultracentrifuge. Since heparin is not absolutely homogeneous as regards molecular size it was not possible to calculate an exact value but a molecular weight of 17 000 was obtained as an average for all the molecules in the preparation.

Heparin is also inhomogeneous in respects other than molecular weight. Several mild physical methods have been employed to divide seemingly pure samples of heparin into components differing in sulphur and nitrogen content and activity. Fractional recrystallization of the brucine salt by Jorpes & Bergstrom (1937) pointed to the association of sulphur content with activity. In the Craig counter-current extractor O'Keef, Russo-Alesi, Dolliver & Stiller (1949) separated heparin into a component having 8.2% S and an activity of 59 units/mg. and one with 13.3% S and an activity of 215 units/mg. By the technique of frontal analysis, Jensen, Snellman & Sylvén (1948) detected three or four components in heparin.

From the facts outlined above, it appears that blood anticoagulant activity is not the property of a unique molecular configuration but is shared by a number of similar molecules occurring in the product ordinarily called heparin. An outstanding feature which has long been recognized is the neces-

sity for the presence of acidic sulphate groups in molecules with anticoagulant activity. The literature contains many examples of sulphuric esters of polysaccharides exhibiting heparin-like activity *in vitro*. Sulphuric esters of dextran (Grönwall *et al.* 1945), cellulose (Astrup & Piper, 1945*a*), inulin (Ingelman, 1946) and alginic acid (Snyder), to mention but a few, have been prepared. Usually these esters have been reported to show one-tenth to one-half of the activity of heparin *in vitro*, but inulin sulphuric ester is claimed to be as active as heparin *in vitro*.

Compounds with blood anticoagulant activity are easily prepared, but when submitted to toxicity tests some active preparations have proved fatal in doses only a few times greater than the minimum dose effective in raising the blood clotting time. Astrup & Piper (1945*a, b*) found that some sulphuric esters of cellulose combined with fibrinogen to form an insoluble complex. They considered such an adverse effect on fibrinogen should not occur with an anticoagulant intended for clinical use.

There appeared to be sufficient scope for systematic variation in the structure of anticoagulant molecules to enable combination with fibrinogen to be minimized while retaining a useful degree of anticoagulant activity. It seemed essential to control two factors: (1) molecular weight; (2) number of sulphate groups.

Considering first the effect of molecular weight, it is desirable to start with a polysaccharide of which the molecular weight can be subjected to controlled variation. On account of its use as a blood plasma substitute (Ingelman, 1949; Bull *et al.* 1949), pure dextran is produced in large quantities and considerable attention has been given to molecular weight control (Ingelman, 1949; Ricketts, Lorenz & Maycock 1950). Grönwall *et al.* (1945) describe three preparations of dextran sulphuric acid ester. Their least toxic preparation was made from dextran of relative viscosity 2.4 in 6% solution. It may be inferred from the viscosity data given by Ingelman & Halling (1949) that this dextran had a molecular weight of about 32 000. The sodium salt of this dextran sulphate contained 12% sulphur, or about 1 sulphate group per glucose unit so the molecular weight of the sodium dextran sulphate would be about

50 000. This is about 3 times the molecular weight of heparin. However, in a subsequent paper, reporting the toxicity of an inulin sulphuric acid ester of molecular weight 14 000, Ingelman (1946) expresses the view that 'molecular weight is not a principal factor in determining toxicity'.

To resolve this point a series of sulphuric esters of dextran differing widely in molecular weight and sulphur content was prepared. The cause of their toxicity was carefully investigated and will be reported separately by Dr K. Walton.

Dextran sulphate with molecules of a certain size was found to be free from toxic effects. The relation between blood anticoagulant activity and number of sulphate groups in molecules of this size was explored.

On the basis of these experiments the preparation of dextran sulphate in a form suitable for clinical trial is reported.

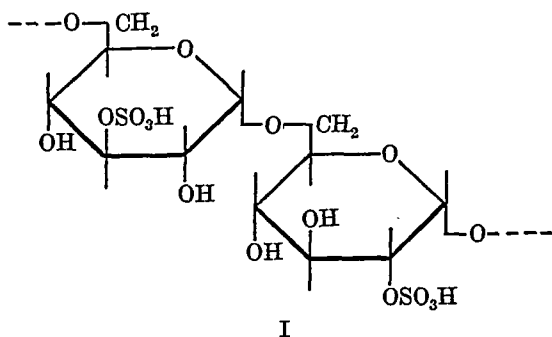
EXPERIMENTAL

Material

The starting material was dextran prepared for use as a plasma substitute (Bull *et al.* 1949). In the course of preparation the dextran of *Leuconostoc mesenteroides* was partially hydrolysed by boiling with 0.075 N-H₂SO₄ until its intrinsic viscosity was between 0.21 and 0.36. Tests to ensure freedom from toxicity and antigenicity were carried out as described by Bull *et al.* (1949).

Methods

Sulphate groups were introduced by treating the finely powdered dry dextran with chlorosulphonic acid and pyridine using the proportions of reagents and conditions of Grönwall *et al.* (1945), except when variations are specifically mentioned, for example in Table 2. The dextran sulphate was isolated as the sodium salt in the way described by these authors.



Sulphur was determined by oxidation of organic matter with Benedict's sulphur reagent, precipitation of inorganic sulphate as benzidine sulphate (Owen, 1936) and titration with standard alkali. A dialysate was carefully tested to ensure the absence of inorganic sulphate from each preparation, so that the sulphur may be regarded as ester sulphate. The sulphur thus determined was assumed to be combined in the way illustrated in the formula I. On this basis Fig. 1 shows the calculated relationship between percentage sulphur in the sodium salt and the number of sulphate groups per glucose unit.

Intrinsic viscosity was obtained from measurements of relative viscosity at several concentrations thus:

$$\eta_{sp.} = \eta_r - 1,$$

$$[\eta] = \lim_{C \rightarrow 0} \frac{\eta_{sp.}}{C},$$

where η_r = relative viscosity, $\eta_{sp.}$ = specific viscosity, $[\eta]$ = intrinsic viscosity.

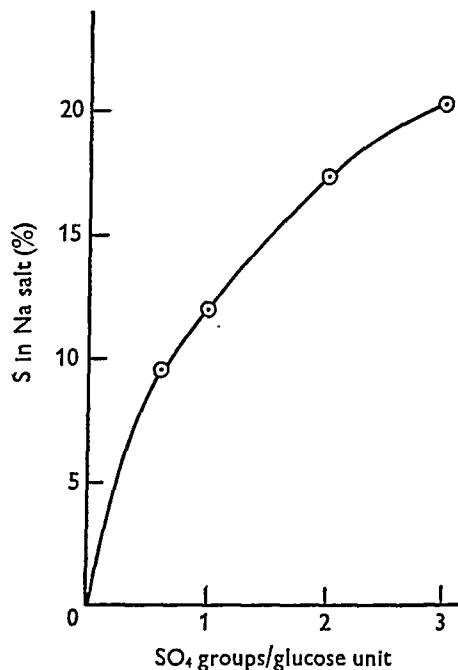


Fig. 1. Showing the calculated relation between sulphur content of sodium dextran sulphate and average number of sulphate groups per glucose unit.

Osmotic pressure was measured in an Adair (1949) type of osmometer and recorded in cm. of solution after correction for capillarity. All measurements were made at 3-4°.

Anticoagulant activity was measured by the method of Kuizenga, Nelson & Cartland (1943) using sheep plasma.

RESULTS

Variation of molecular weight

Three main types of dextran sulphate, designated by the letters *D*, *E* and *I*, were prepared. The *D* and *I* series were from unfractionated hydrolysates of the intrinsic viscosity quoted in Table 1. The *E* series was prepared from the low molecular 50 % of a hydrolysate with intrinsic viscosity 0.24; this fractionation was made to minimize any overlap in molecular composition between the *D* and *E* series.

If the relation between intrinsic viscosity and molecular weight determined by Ingelman & Halling (1945) be applied to the dextran used in these experiments the average molecular weight of the

dextran used for the *D* and *E* types of dextran sulphate is about 200 000 and 20 000 respectively. Allowing for the introduction of, say, 1.3 sulphate groups per glucose unit, the average molecular weight of the *D* and *E* types of dextran sulphate would be very approximately 300 000 and 30 000 respectively. The only estimate for the *I* type of dextran sulphate that can be obtained from the data given by Grönwall *et al.* (1945) is a figure of less than 20 000, allowing for the introduction of 1.3 sulphate groups per glucose unit.

Table 1. *Properties of dextran sulphates prepared from three dextrans differing widely in molecular weight*

Intrinsic viscosity of dextran	Serial	S in Na salt (%)	SO ₄ groups/glucose unit	Activity units/mg.
0.36	<i>D</i>	14.6	1.4	—
0.36	<i>D</i> 2	17.8	2.1	—
0.36	<i>D</i> 3	15.4	1.6	15
0.12	<i>E</i>	21.5	3.0	—
0.12	<i>E</i> 1	13.7	1.3	15
0.02	<i>I</i>	13.5	1.2	—
0.02	<i>I</i> 2	15.2	1.5	15

Evidence that the three types of dextran sulphate differ in average molecular weight was obtained from viscosity and osmotic pressure measurements. Viscosity was measured in 0.066 M-phosphate buffer at pH 7. Fig. 2 shows specific viscosity divided by concentration plotted against concentration for preparations *D*3, *E*1 and *I*4 (see Tables 1, 3). Extrapolation directly and using the logarithmic method mentioned by Bawn (1948) gave identical values for the intrinsic viscosity. From this graph the intrinsic viscosity of *D*3 may be read as 0.69, *E*1 as 0.20 and *I*4 as 0.04. Similar measurements in 0.9% (w/v) sodium chloride showed: *E*1, 0.18; *I*4, 0.03; and *I*3, 0.03; heparin sample *A*, 0.21; heparin sample *B*, 0.12.

The osmotic pressure of 2% (w/v) *E*1 and 1% (w/v) *I*3 in 0.9% sodium chloride solution was measured. The solution outside the membrane was tested with toluidine blue but no metachromatic reaction was obtained in a test which was known to be sensitive to less than 10 µg./ml. The molecular weights calculated from the equilibria were *E*1, 35 000 and *I*3, 8000, but several such osmometers are necessary to provide accurate estimates of number average molecular weight. It may be inferred from these data that the molecular weight of *I*3 is considerably less than that of *E*1. Using two osmometers and a more refined technique, Mr D. Sutcliffe has obtained a figure of 7300 ± 500 for the preparation *I*4.

Biological testing of the preparations showed that the *I* series of dextran sulphates was free from toxic

effects and might be therapeutically useful. Dextran sulphates of the *E* and the *D* series were found to be toxic.

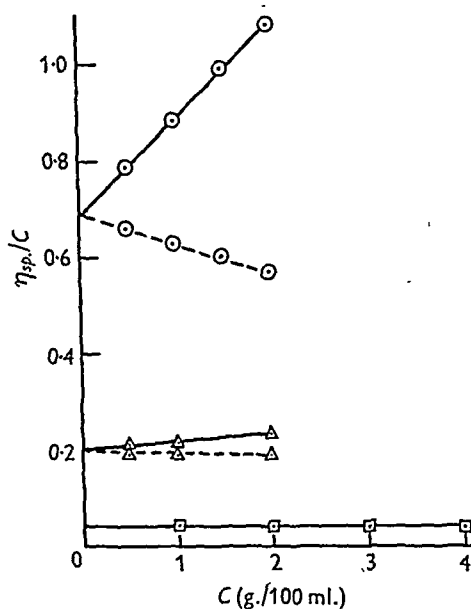


Fig. 2. Showing direct (—) and logarithmic (---) extrapolation to obtain intrinsic viscosity of dextran sulphate preparations *D*3, ○; *E*1, △ and *I*4, □.

Variation in number of sulphate groups

Several dextran sulphates differing in sulphur content were prepared from a single batch of dextran which had an intrinsic viscosity of 0.04.

Table 2. *Properties of dextran sulphates prepared from the same batch of dextran but differing in sulphur content*

ClSO ₃ H ml./10 g. dextran	Serial	S in Na salt (%)	SO ₄ groups/glucose unit	Activity units/mg.
14.6	<i>I</i> 7	16.3	1.7	15
13.3	<i>BE</i>	15.9	1.6	15
12.0	<i>BD</i>	14.2	1.3	15
10.6	<i>BC</i>	14.3	1.3	15
9.3	<i>BB</i>	11.8	1.0	3.75
—	<i>AO</i>	9.2	0.6	<3

Preliminary experiments indicated that alteration in the sulphur content was not achieved by decreasing the proportion of chlorosulphonic acid from 44 ml./10 g. dextran as quoted by Grönwall *et al.* (1945) to 14.6 ml./10 g. dextran, but was obtained between this value and 9.3 ml./10 g. dextran. Table 2 shows this effect, together with the sulphur content and activity of the resultant dextran sulphate. Preparation *AO*, also shown in Table 2, was made by adding powdered dextran to the melted crystalline complex of chlorosulphonic acid and pyridine (Sobel, Drechter & Natelson, 1936) and

Table 3. *Data for large-scale preparations of dextran sulphate*

Intrinsic viscosity dextran	Weight dextran (g.)	Serial	Yield Na salt (g.)	S in Na salt (%)	SO ₄ groups/ glucose unit	Activity units/mg.
0.02	60	I3	88	17.1	1.9	15
0.03	60	I4	115	17.1	1.9	15
0.03	30	I5	41	17.1	1.9	—
0.05	30	I6	41	16.5	1.8	—
0.04	60	I7	80	16.3	1.7	15

isolating the product in the usual way. The relation between the number of sulphate groups per glucose unit and activity reveals a sharp increase to the maximum activity between 1 and 1.3 sulphate groups per glucose unit.

For comparison, the provisional International Standard for heparin contains 12.45 % sulphur in the sodium salt and has an activity of 130 units/mg. Heparin sample *A* contained 9.3 % S and sample *B* contained 8.6 % S.

Preparation of a non-toxic dextran sulphate

The foregoing experiments established the conditions for the preparation of an active non-toxic dextran sulphate. In view of the association of toxicity with increasing molecular weight the larger molecules were removed by fractional precipitation before introducing the sulphate groups. This precaution was intended to prevent significant amounts of toxic dextran sulphate accumulating when very large doses were given over a long period of time. A full description of the preparation of the dextran sulphate submitted to clinical trial will now be given.

To 3 l. of a 6.2 % (w/v) solution of a partial hydrolysate of *L. mesenteroides* dextran with intrinsic viscosity 0.25, 300 ml. $N-H_2SO_4$ were added and the solution boiled under reflux for 4 hr. After cooling, the solution was neutralized with $N-NaOH$, the final volume being 3.6 l. Acetone (3 l.) was added, and after standing overnight the syrupy precipitate containing the larger molecules was separated. A further 6 l. of acetone were added, the syrup was separated, poured into ethanol, ground to a powder, washed with dry ether and dried *in vacuo* over P_2O_5 . About 100 g. were obtained; the intrinsic viscosity was 0.02.

To 400 ml. dry pyridine, 88 ml. chlorosulphonic acid were added drop by drop with vigorous stirring. During the addition the flask was cooled in a mixture of solid CO_2 and ethanol. The temperature was then raised to 65° when most of the pyridinium salts dissolved. Finely powdered dextran, 60 g., was added and dispersed in the reaction mixture by vigorous stirring. The temperature was maintained at 65–70° for 4 hr.

After cooling, 1.5 l. of crushed ice and sufficient 40 % (w/v) $NaOH$ were added to turn the mixture dark red in colour and cause separation of the pyridine as an upper layer. The lower layer was diluted to 2 l. and brought to 37° when 2 l. of ethanol were added. The precipitated syrup was allowed to settle for 10 min. only at 37° to avoid crystallization of Na_2SO_4 . The syrup was then separated,

redissolved in 850 ml. water and similarly precipitated with 850 ml. of ethanol. The precipitation was repeated again from 640 ml. of solution and an equal volume of ethanol. The final syrup was dissolved in 800 ml. of water, neutralized with HCl , dialysed against running water and treated with charcoal at 50°. The pH of the filtrate was adjusted to 7–7.5 with $NaOH$ and the solution concentrated under reduced pressure to 500 ml. Acetone (750 ml.) was added, and the precipitated syrup was poured into ethanol, ground to a powder, washed with ether and dried *in vacuo* over P_2O_5 . This preparation was designated I3. Details of this and a number of similar preparations are summarized in Table 3.

Dextran sulphates of the *I* series decompose on boiling in aqueous solution with the formation of inorganic sulphate and reducing substances, a point of some importance in connexion with the sterilization of solutions for intravenous administration. For example, after autoclaving a neutral 20 % (w/v) solution of I4 at 10 lb. steam pressure for 20 min., one-third of the sulphur was liberated as sulphuric acid and the solution was found to reduce Fehling's reagent strongly. Some of the dextran sulphate which had not decomposed was recovered and found to contain 13.5 % S and to be about as active as I4.

It was found that this decomposition could be prevented by buffering the solution. The following mixture has proved satisfactory: dextran sulphate, 20 g.; sodium chloride, 0.72 g.; sodium bicarbonate, 0.24 g.; water, 100 ml. A solution of this composition had an initial pH of 8.3, and was autoclaved at 10 lb. for 10 min. without the appearance of inorganic sulphate or reducing substances, and without appreciable loss of activity. The final pH was 6.7 and the solution approximated to the isotonicity required for intravenous use.

DISCUSSION

With dextran as with other polysaccharides, blood anticoagulant activity is readily obtained by the introduction of sulphate groups. Interest therefore centres on whether any such compounds are sufficiently non-toxic for clinical use. A preliminary communication of the results of the biological investigation which proceeded in parallel with the experiments reported here has been given by Walton (1951) and a full account is in course of preparation. Toxicity was found to increase with molecular weight.

It is therefore important to consider the evidence relating to the molecular weight of the three types of dextran sulphate preparation used in the biological investigation. The intrinsic viscosity of the dextrans and of the dextran sulphates derived from them indicate corresponding differences in molecular weight. The critical distinction in toxicity is between the *E* type and the *I* type and here the osmotic pressures recorded support the contention that the *I* type is of considerably lower molecular weight than the *E* type, being of the order of 7000.

The anticoagulant activity expressed in units/mg. appears to be independent of the molecular weight but depends on a certain minimum number of sulphate groups per glucose unit. At 1.0–1.3 sulphate groups per glucose unit the sharp increase of activity to its maximum value is indicative of the appearance of some critical pattern of groups in the molecular structure. Heparin has greater activity with considerably fewer sulphate groups in the molecule. From the biological investigations evidence was obtained that the activity of heparin and dextran

sulphate was qualitatively identical, in spite of the sevenfold difference in the weight corresponding with a given amount of anticoagulant activity.

SUMMARY

1. Molecular weight determines the toxicity of dextran sulphate. Molecules smaller than a critical size are not toxic.

2. Maximum blood anticoagulant activity is attained when the number of sulphate groups exceeds an average of 1.3 per glucose unit.

3. Preparation of a potentially useful anticoagulant drug is described.

It is a pleasure to acknowledge here the close and fruitful collaboration of my colleague, Dr Kenneth Walton, whose account of the biological aspects of our investigation will appear elsewhere, and to thank Prof. J. R. Squire for his help and encouragement. Thanks are also due to Prof. M. Stacey, F.R.S., for placing at my disposal the facilities of his Department for the larger scale preparations of dextran sulphate.

I am grateful to Mr M. Hall, for his invaluable help in the laboratory, and to Messrs Dextran Ltd. for gifts of dextran.

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The Occurrence of Substituted Uric Acids in Human Urine

By E. A. JOHNSON

Medical Research Council Spectrographic Unit, London Hospital, London, E. 1

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In the course of work on the separation of purines and pyrimidines from biological materials, purine fractions obtained from human urine have been examined. This work is not complete, but as it is being interrupted the results so far obtained are given here.

The purine fractions employed were obtained by ammoniacal silver precipitation of the urine after

phosphate removal using the reagents described by Snell & Snell (1937), and separation was effected on columns of the ion-exchange resin Zeo-Karb 215 by methods similar to those used by Cohn (1949) for the separation of bases from nucleic acids. Uric acid was readily separated from the xanthines, but between these, other well-defined fractions were obtained having ultraviolet absorption spectra scarcely

distinguishable from those of uric acid. These materials were isolated, though in exceedingly small quantities, and were evidently methyluric acids. Confirmation of this was obtained by the complete cessation of the excretion of these materials when the subject abstained from tea, coffee and cocoa, products all containing methylxanthines, and by their appearance in the urine again after ingestion of caffeine and theophylline.

This work therefore extends that of Myers & Hanzal (1946), Buchanan, Block & Christman (1945), Buchanan, Christman & Block (1945) and other workers, well summarized in the papers of these authors, on the metabolism of the methylxanthines.

EXPERIMENTAL

Separation and chromatography

Several methods of separating purines from urine were briefly examined; of precipitation methods none was found to have any marked advantage, at least qualitatively, over the ammoniacal silver method. The CHCl_3 extraction method of Fisher, Algeri & Walker (1949) was found to yield three methylxanthines, probably theobromine, theophylline or paraxanthine, and caffeine, and at least three other unidentified compounds. The products from these separation methods were examined by paper chromatography employing as solvent *n*-butanol- NH_3 (Hotchkiss, 1948) by which the methylated xanthines are well separated (see also Markham & Smith, 1949). For separation of uric acids a single-phase solvent mixture containing *n*-butanol, ethylene glycol monomethyl ether, acetic acid and water in the proportions 7:7:2:4 (v/v) was found satisfactory. Chromatograms were usually run on paper strips 1 in. (2.5 cm.) wide by upward displacement in boiling tubes 12 in. long and 1.5 in. internal diameter (30 × 3.7 cm.). The tubes were held vertically with the solvent mixture in the bottom. The mouth of each tube was closed with a rubber stopper through which passed a glass rod ending in a hook on which the paper was carried. Two lanes could be run quite satisfactorily on such strips when direct comparison was required. R_F values varied appreciably with the ambient temperature, and values quoted here for the substituted uric acids are given as relative R_F 's, i.e. as ratios to that of uric acid itself. In the above solvent at about 20°, uric acid has an R_F value of 0.25. Spots were located in ultraviolet light (Holiday & Johnson, 1949).

The largest column of Zeo-Karb 215 resin employed was 21 cm. long and 1.8 cm. in diameter, and contained 12 g. finely ground resin (settling time 1–10 min.) mixed with an equal weight of Hyflo-Supercel. This was recycled a number of times with KOH or NH_3 and HCl, and finally equilibrated with 0.5N-HCl. Quantities of up to 2 l. of urine were treated with magnesia mixture (Snell & Snell, 1937) and NH_3 to precipitate phosphate, centrifuged, and the supernatant treated with a slight excess of ammoniacal silver reagent. After standing in the dark for at least 1 hr., the precipitate then formed was spun down, washed first with very dilute ammonia and then with water, suspended in a little water and N-HCl added until the pH was about 2. The volume was made up to about 20–30 ml., the mixture heated to boiling and filtered hot. The solution was allowed to crystallize

overnight, depositing principally uric acid, and the supernatant poured on to the column. Even had it been practicable to charge the column with a volume sufficient to retain all the uric acid in solution, it seemed very probable that this would have tended to crystallize out in the column during the separation, especially in view of the solubilizing effects referred to later. 0.5N-HCl was used as eluent, and fractions of 4.04 ± 0.02 ml. were collected by a siphon-operated automatic collector. The optical densities of the fractions were measured at a wavelength of 285 m μ . with a Unicam SP 500 spectrophotometer. Purine from 2 l. of urine obtained as described above tended to overload the column, in that considerable overlap between fractions occurred, but from previous experience it was readily possible to separate the groups containing the individual acids. Smaller quantities gave excellent discrimination, as shown in Fig. 3. Xanthines were retained on the column until all uric acids had been eluted. They could be displaced by increasing the acid strength to 2N, but there was little discrimination within the group. At least four xanthines were shown to be present in this fraction by paper chromatography. The uric acids were isolated from their solutions in 0.5N-HCl by running these down a column of the anion-exchange resin Duolite A. 2 in the free base form. The capacity of the column for HCl was chosen to be slightly greater than that present in the solution, and the uric acid was then displaced by 0.01N-HCl. The resulting solution was evaporated to dryness under reduced pressure. Earlier experiments in which the 0.5N acid solution was evaporated directly indicated that some loss was incurred, and although the column method also involved some losses it was thought that it involved less chance of decomposing the uric acids.

Identification

Absorption spectroscopy. Probably the most straightforward and generally useful method of identification for purines in very small quantities is that of ultraviolet absorption spectroscopy. The absorption spectra of the three uric acid fractions (referred to hereafter as I, II and III) obtained from urine differed, however, from one another very little (Table 1). Examination of the spectra of a number of methyl-substituted uric acids originally made by Prof. H. Biltz and kindly provided by Prof. D. Keilin, F.R.S., showed that those substituted in the 3- and in the 9-positions differed significantly and characteristically from uric acid itself, while 1- and 7-substituted uric acids showed very much smaller differences. The absorption spectra of uric acid itself at various pH values have been published by a number of authors, including Holiday (1930), Fromhertz & Hartmann (1936) and Stimson & Reuter (1943), but it is felt that improved techniques justify republication (Fig. 1, Table 1). These curves were obtained on an automatic recording spectrophotometer (Holiday & Sutton, to be published) with point density checks on a Unicam SP 500 spectrophotometer. Two commercial samples of uric acid were employed, purified by combinations of three methods: solution in conc. H_2SO_4 and fractional precipitation by dilution, precipitation from the lithium salt, and recrystallization from water or dilute hydrochloric acid. The products did not differ significantly.

Arsenophosphotungstic acid colour reaction. The Benedict arsenophosphotungstic acid colour reaction also offered a valuable method of distinguishing between the various substituted uric acids. Unfortunately, the original method of

Table 1. *Ultraviolet absorption of uric acid and of some methyluric acids*

(Wavelengths given in $m\mu$. Values given for uric acid and for 7-methyluric acid are believed to be accurate in wavelength to 0.5 $m\mu$. and in molecular extinction coefficient ϵ to 0.01×10^4 ; for the other compounds values are given for purposes of comparison and are subject to revision, particularly those in alkaline solutions.)

Compound	pH 3			pH 8-8.5			pH > 12		
	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$
Uric acid	284	—	1.21	291.5	—	1.24	294.5	—	1.35
	—	255	0.365	—	260.5	0.26	—	262	0.25
	230.5	—	0.87	235	—	0.985	219.5	—	2.54
	—	212	0.52	—	218.5	0.59	—	—	—
7-Methyluric acid	286	—	1.14	293	—	1.22	296.5	—	1.31
	—	258	0.39	—	263	0.30	—	262	0.255
	234	—	0.87	237	—	0.97	222	—	2.37
	—	213	0.50	—	219	0.595	—	—	—
1:7-Dimethyluric acid (fraction III)	285.5	—	1.10	—	—	—	295.5	—	1.27
	—	258	0.435	—	—	—	—	263.5	0.33
	234	—	0.84	—	—	—	222	—	2.60
	—	220	0.64	—	—	—	—	—	—
1-Methyluric acid	283.5	—	1.14	—	—	—	292.5	—	1.23
	—	253.5	0.39	—	—	—	—	260.5	0.30
	231	—	0.79	—	—	—	217.5	—	2.14
	—	218	0.66	—	—	—	—	—	—
3-Methyluric acid	287	—	1.10	—	—	—	292.5	—	1.49
	—	257	0.31	—	—	—	—	257.5	0.24
	232	—	0.79	—	—	—	214	—	1.84
	—	215	0.49	—	—	—	—	—	—
1:3-Dimethyluric acid	285.5	—	1.16	—	—	—	294	—	1.69
	—	258	0.41	—	—	—	—	260	0.335
	234	—	0.88	—	—	—	214.5	—	1.90
	—	221	0.77	—	—	—	—	—	—

Benedict & Franke (1922) and the adaptation used by Buchanan, Block & Christman (1945) are not suitable for application to microgram quantities such as are eluted from paper chromatograms, and yet another modification had to

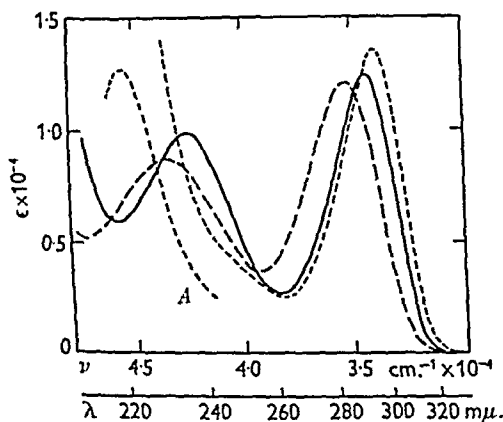


Fig. 1. Ultraviolet absorption spectra of uric acid. —, pH 3; — —, pH 8; - - - -, pH > 12. For short wavelength peak at pH > 12 (curve A), values of ϵ should be multiplied by 2.

be worked out. It was most convenient to measure the ratio between the colour developed and the peak optical density in alkaline solution, giving a value characteristic for each compound without requiring any determination of absolute quantity present. The colour development was carried out as follows. To 2.5 ml. uric acid solution was added 0.5 ml. of an

aqueous solution containing 15% Na_2CO_3 and 15% urea, 0.5 ml. 10% aqueous NaCN , and 0.5 ml. arsenophosphotungstic acid reagent (Benedict & Franke, 1922). The solution was then 'seeded' with a little of the precipitate which always forms after a variable delay, and left to stand for 30 min. It was then centrifuged and the optical density of the supernatant measured against that of a reagent blank at the absorption peak 700 $m\mu$. in the Unicam SP 500 spectrophotometer. This value was divided by the optical density at 295 $m\mu$. given by the same amount of the uric acid in 40 ml. aqueous solution at a pH > 11.5. 'Colour ratios' obtained in this manner have proved quite reproducible, and not significantly affected by the age of the reagents. The order of the colour ratios obtained in this manner agrees, for those acids used, with that given by Buchanan, Block & Christman (1945). All the acids substituted in position 7 give very low values. The reaction is also applicable in simplified form to paper chromatograms. These are lightly sprayed with saturated Na_2CO_3 solution, and then with the arsenophosphotungstic acid solution diluted 1 in 3 with water.

Acid strength determinations. It would be expected that substitution of the possibly ionizable hydrogen atoms in the uric acid molecule by methyl groups would affect the acid strength. Biltz (1936) gives the order of acid strength of the four hydrogen atom positions as $9 > 3 > 1 > 7$, and states that uric acids not substituted in either the 9- or the 3-positions have very similar acid strengths. As a further check therefore on the substitution positions of the acids isolated from urine, spectrophotometric titrations were carried out on them and also on uric acid, 7-methyluric acid and 3-methyluric acid. The change with pH of optical density at a suitable wavelength was determined; buffers were not used, but, using a glass electrode system, solutions in very dilute acid or

alkali were titrated with very small volumes of strong alkali or acid, so that changes in volume were negligible. The wavelengths chosen were 244 and 302 $m\mu$., of which the second proved the more suitable. Curves of the type shown in Fig. 2 were obtained, from which pK values could be estimated with an accuracy of about ± 0.1 pH unit.

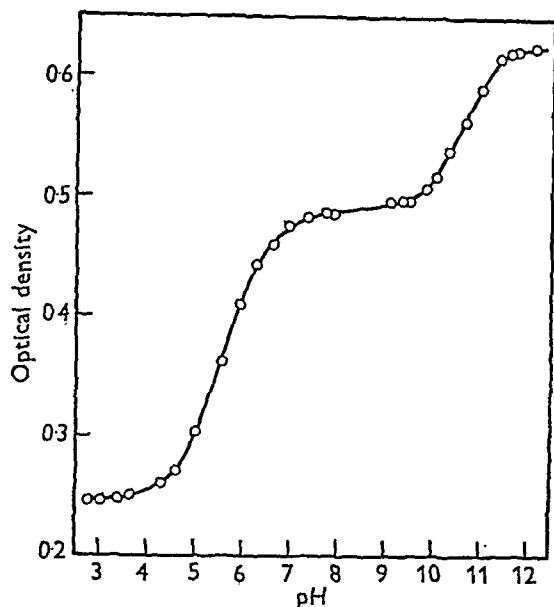


Fig. 2. Relation between optical density and pH at 302 $m\mu$. for fraction II of urinary uric acids.

Crystallization. The isolated uric acids were recrystallized from water and possessed distinguishable crystalline forms. Myers & Hanzal (1946) give some data on the solubilities of methyluric acids which, as with the xanthines, increase with an increasing degree of methylation. It was observed that the substituted acids from urine were very much more soluble in hot water than uric acid itself.

RESULTS

The purines from normal urine samples with no dietary precautions yielded by chromatography on a Zeo-Karb 215 resin column three fractions, as shown in Fig. 3 *b*, all showing very similar ultra-violet absorption spectra. The first of these was usually present in the greatest quantity, and was shown by paper chromatography, colour ratio and crystalline form to be uric acid itself.

Fraction II invariably proved on isolation to be heterogeneous. The first crystallization of the evaporation residue gave an aggregate of colourless crystals mixed with yellowish spherical granules, with very variable colour ratio, 0.9–2.2, compared with 2.9 for uric acid itself. On paper chromatography the material streaked badly, but gave a principal spot exactly corresponding in position to that of 7-methyluric acid, with a relative R_f of 1.4. Colour reaction on the paper was largely confined to the streak behind this spot and to material left at the

starting point. On recrystallization from water the yellowish material remained largely undissolved, and the product which was visually free from it formed very characteristic irregular stellate clusters

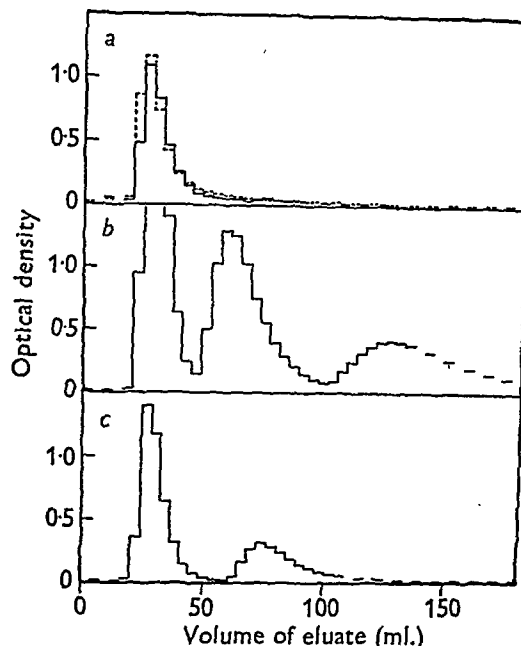


Fig. 3. Elution of urinary uric acids from a Zeo-Karb 215 resin column. (a) Full line: after 2 days' abstinence from foods and beverages containing methylated xanthines; broken line: after 4 days' abstinence. (b) After administration of 400 mg. caffeine. (c) After administration of 250 mg. theophylline.

of blades. On paper chromatography after recrystallization the colour-forming streak was much reduced, and the colour ratio was also reduced in solution to 0.47. Absorption spectra of this material were determined at pH values of 3 and > 12 , at a concentration of 0.245 mg. in 20 ml. They were not distinguishable from those of 7-methyluric acid (Table 1) in shape and band positions, and values were obtained for $E_{1\%}^{1\text{cm}}$ of 553 at 286.5 $m\mu$., pH 3, and 627 at 296.5 $m\mu$., pH > 12 , compared with corresponding values of 625 and 720 for 7-methyluric acid. Further purification was not attempted, since too little of the material was available.

Fraction III appeared to be quite homogeneous. It was quite soluble in hot water, from which it crystallized in plates, though sometimes small needles appeared singly or in radiating clusters. The colour ratio was 0.175, comparing closely with the value of 0.15 obtained for 7-methyluric acid, but the relative R_f was 1.8. Absorption spectra were determined at pH 3 and > 12 on solutions of concentration 0.303 mg. in 30 ml. The results are given in Table 1; the reasons for considering this compound to be 1:7-dimethyluric acid are given in the discussion.

Table 2. *Acid strengths, colour ratios and relative R_F values of uric acid and some methyl derivatives*

(Colour ratios and acid strengths are determined by the methods described in the text. The R_F values are relative to that of uric acid, using the butanol-methyl cellosolve-acetic acid solvent.)

Compound	Colour ratio	Relative R_F	pK_1	pK_2
Uric acid	2.9	1.0	5.4	10.6
1-Methyluric acid	3.4	1.5 (streaks)	—	—
3-Methyluric acid	1.6	0.9 (streaks)	6.2	>11.4 (?)
7-Methyluric acid	0.15	1.4	5.5	10.6
1:3-Dimethyluric acid	1.7	2.0	—	—
1:7-Dimethyluric acid (fraction III)	0.175	1.8	5.7	10.9

Values for the pK 's of these compounds were determined spectrophotometrically, and it was found that the differences between them were very small, scarcely exceeding the estimated error of ± 0.1 pH unit. Fig. 2 shows the curve obtained for fraction II, from which $pK_1 = 5.6$, $pK_2 = 10.6$. This particular curve was chosen since it is continuous; in other cases, the concentrations for pK_1 differed from those used for the pK_2 determinations, but results were very similar. The other values are given in Table 2; those obtained for uric acid differ from the results of His & Paul (1900) quoted by Holiday (1930), which correspond to values of 5.7 and 8.7.

Since it appeared probable that methyl groups constituted the substituents in these uric acids and that they were therefore metabolic products from methylated xanthines, some runs were carried out on purines from urine obtained after 2 and 4 days' abstinence from foods or beverages containing such compounds (Fig. 3a) and also after oral administration, with similar diet, of 400 mg. caffeine (Fig. 3b) and 250 mg. theophylline (Fig. 3c). In each case the urine samples of 25 ml. were treated similarly, and the purine solutions in 4.3 ml. of very dilute hydrochloric acid were run on a column 14 by 1.3 cm. containing 5 g. resin and a similar weight of Hyflo-Supercel, which had been in use for some time and gave quite reproducible results. It can be seen that if consumption of methylxanthines is avoided, only one uric acid fraction is obtained, that of uric acid itself. After the administration of caffeine the pattern found corresponds exactly to that of the normal urine samples previously investigated, with two methyluric acid fractions in addition to that of uric acid, whereas the metabolism of theophylline yields only one methyluric acid fraction under the conditions described.

A phenomenon which may be observed in Fig. 3, and which was conspicuous throughout this work, is the increased amount of uric acid which may remain dissolved in a given volume of water or dilute hydrochloric acid in the presence of methyluric acids, or possibly of methylxanthines. This solubilizing effect was particularly conspicuous when larger quantities of purine materials were dealt with; uric

acid fractions from the column proved to be supersaturated and crystallized out on standing from volumes of solution greater than those originally charged to the column, which had themselves been allowed to crystallize to equilibrium. Examination by paper chromatography of the material crystallizing out of solutions from the decomposition of the silver purines showed that although this was principally uric acid, substituted uric acids and xanthines were also present.

A synthetic mixture of uric acid, 3-methyluric acid and 3:7-dimethyluric acid was also run on the smaller column. 3-Methyluric acid was eluted immediately after uric acid, the peaks being at 28 and 45 ml., and the peak of the 3:7-dimethyluric acid fraction came at 115 ml. This experiment was carried out in the earlier stages of the work to determine the behaviour of known methyluric acids on the column. These particular acids were chosen since they could readily be distinguished by absorption spectra or colour ratios.

DISCUSSION

The results obtained are essentially qualitative. Myers & Hanzal (1946) state that the methyluric acids are all precipitated on standing by both ammoniacal silver magnesium mixture and by Folin's silver lactate-lactic acid solution. Buchanan, Christman & Block (1945), however, state that under the conditions used in their work 1:3-dimethyluric acid and 3-methyluric acid were not precipitated by ammoniacal silver. In the present work no evidence of the presence of any 3-substituted acids was found. Further possible segregation was caused by solubility limitations, which made it impracticable to employ total purine fractions for analysis on resin columns. The effect here, however, was one of relative enrichment of the more soluble methylated acids at the expense of uric acid, though it was found that some methyluric acids were left in the crystallized material.

Of the separated fractions, fraction II may reasonably be inferred to consist of a mixture of 7-methyluric acid and 1-methyluric acid with the

yellowish granules as a possible third component. Both 7- and 1-methyluric acid have absorption spectra closely resembling that of uric acid (Table 1), and mixtures of the two of varying composition could yield the colour ratios reported, corresponding to an increase in the proportion of 7-methyluric acid in the product from recrystallization. 1-methyluric acid tends to streak during paper chromatography, but the principal spot is found at almost the same position as 7-methyluric acid (Table 2). After ingestion of theophylline (Fig. 3c) no trace is observed of 3-methyluric acid, which would be eluted immediately after uric acid, and the single methyluric acid fraction must be attributed, therefore, to the 1-methyl or 1:3-dimethyl compound; absorption band positions and relative intensities indicated that it was 1-methyluric acid. The elution peak of the corresponding fraction obtained after ingestion of caffeine lies closer to that of uric acid. This fraction presumably contains the two overlapping elution bands of 7- and 1-methyluric acids.

Fraction III, if derived from the oxidation, with or without demethylation, of methylxanthines, might possibly then be 1:3-, 1:7- or 3:7-dimethyluric acid, or 1:3:7-trimethyluric acid. Since this fraction is not obtained from theophylline, the first is improbable and is in any case excluded, since fraction III gives a very low colour. The last two may also be eliminated since their absorption spectra are quite distinct from that of fraction III. Unfortunately, no sample of 1:7-dimethyluric acid was available for direct comparison. The absorption spectrum, however, was scarcely distinguishable from that of 7-methyluric acid, and it had been observed that the spectra of the corresponding 7-methyl and 1:7-dimethylxanthines are, in fact, practically indistinguishable, and give almost identical molar extinction coefficients. Allowing for the very small amount, 0.303 mg., on which the determination was carried out, the values obtained for fraction III show good agreement with those for 7-methyluric acid (Table 1).

The almost identical acid strengths of uric acid, 7-methyl and the presumed 1:7-dimethyluric acids (Table 2) are in agreement with the statement by Biltz (1936) that all such acids not substituted in the 3- or 9-positions will have similar strengths, and it was found that 3-methyluric acid is significantly weaker. It appears from these results that the separation obtained on resin columns does not depend on acid strength. It does, however, bear some relation to the position and degree of methylation; so far as has been determined the order of elution, after uric acid, is 3, 7, 1, 3:7, 1:7.

Little can be added on the basis of this work to the deductions summarized by Myers & Hanzal (1946) concerning resistance to demethylation in various positions. The results give no reason to dispute the general conclusion that methyl groups in the 3-position are the least stable, and provide definite evidence that demethylation also occurs in both the 1- and 7-positions.

SUMMARY

1. Substituted uric acids have been isolated from the urine of a human subject on a normal diet.

2. The positions of substituent groups have been determined by comparison with known synthetic methyluric acids, using spectroscopic and other methods. Results indicate that these isolated compounds are also methyluric acids substituted in the 7-, 1-, and 1:7-positions.

3. These compounds have been shown to be metabolic products of exogenous methylxanthines, i.e. caffeine, theobromine or theophylline.

4. Demethylation during metabolism of these compounds can occur in the 3-, 1- and 7-positions, but no definite order of stability has been demonstrated.

The author wishes to express his appreciation to Dr E. R. Holiday and Dr G. H. Beaven for their interest and encouragement during this work.

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Comparative Studies of 'Bile Salts'

4. BILE SALTS OF THE EUROPEAN FROG, *RANA TEMPORARIA*

By G. A. D. HASLEWOOD
Guy's Hospital Medical School, S.E. 1

(Received 2 October 1951)

Previous studies of the bile salts of frogs and toads have been carried out on two species only, and entirely by Japanese workers. Okamura (1928, 1929) isolated 'bufocholane', m.p. 236°, and an acid described as 'bufodeoxycholic acid' from the winter bile of the toad, *Bufo vulgaris* (Jap.). Taurine was also separated from the bile. Makino (1933) obtained from the bile of the same species an alcohol, m.p. 230°, described as tetrahydroxybufostane, $C_{27}H_{48}O_4$, which showed positive Pettenkofer and Hammarsten tests. Trihydroxybufosterocholenic acid, $C_{28}H_{46}O_5$, was described by Shimizu & Oda (1934) as occurring in the toad bile and this was converted by Shimizu & Kazuno (1936b) to bisnorcholic acid; it was thus shown to belong to the cholane series and to have in its molecule three hydroxyl groups in the same position as those in cholic acid. Trihydroxyisosterocholenic acid, $C_{28}H_{46}O_5$, was described by Shimizu & Kazuno (1936a) and this was also converted by Shimizu & Kazuno (1937) to bisnorcholic acid. Kazuno (1940) published an account of a careful and detailed study of that part of the (acidified) bile of the Japanese toad from which the above-mentioned acids, apparently occurring in the unconjugated form, had been removed by ether extraction. The chief constituent was a sulphate, m.p. 197°, which by alkaline hydrolysis yielded pentahydroxybufostane, $C_{28}H_{50}O_5$, m.p. 172°. About 10 g. of this substance were isolated from 4000 gall-bladders (5 l. of bile), in one, and 20 g. from 8500 toads (10 l. of bile) in a second experiment. Pentahydroxybufostane was extensively investigated and its triacetate, m.p. 119°, converted in a yield of about 10 % to cholic acid, which was identified as the acid itself and as its ethyl ester. However, as in the case of the acids, conclusive proof of the C_{28} formula was not given, and it rests only on analytical figures. A neutral compound apparently identical with the alcohols of Okamura (1928) and Makino (1933) was also isolated after alkaline hydrolysis of bile fractions and was renamed tetrahydroxynorbufostane, on the assumption that its molecule contained 27 carbon atoms. A third neutral substance, m.p. 175°, was stated to occur in the unconjugated state in the bile: it was called tetrahydroxycholane, and assigned a C_{24} formula. Like pentahydroxybufo-

stane, it gave positive Pettenkofer and Hammarsten reactions, indicating OH groups probably at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$ in the steroid nucleus.

Bile of the frog *Rana catesbiana* was examined by Kurauti & Kazuno (1939), who isolated cholesterol, an acid (named trihydroxybisnorsterocholanic acid) giving a negative response in the Hammarsten reaction and also the sulphate, m.p. 178°, of a so-called tetrahydric alcohol. By alkaline hydrolysis of this latter substance there was obtained an unsaturated alcohol of m.p. 177°, which was quite arbitrarily given a C_{21} formula and named trihydroxycholene. The acetate (? diacetate) had m.p. 180°. Mabuti (1941) investigated the trihydroxybisnorsterocholanic acid of Kurauti & Kazuno and concluded that it also had OH groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$ as in cholic acid.

The above work showed that further examination of amphibian bile was likely to yield results of much interest and the present report is of a preliminary investigation of the bile salts of *R. temporaria*.

RESULTS

An interpretation of the experimental findings is given in Fig. 1. The chief constituent of the bile was a sulphate ester, readily obtained as the sodium salt (I) as previously described (Haslewood & Wootton, 1950). If the (probably) pentahydric alcohol with which the sulphate is esterified is called 'ranol' and the isolated bile salt 'sodium ranol sulphate', the chief product of acid hydrolysis, as carried out, appeared to be ranol (II) itself, not obtained crystalline, but easily converted by mild acetylation in fair yield into a tetraacetate (IV), which could be oxidized to a monoketone (V). Alkaline hydrolysis of ranol sulphate, on the other hand, led to a mixture from which it was not, except in one experiment, possible to isolate the above tetraacetate. However, there was obtained after partial acetylation a mixture of crystalline acetates, the analysis of one of which (VI) suggested that the main product of alkaline hydrolysis, as usually carried out, was a substance derived from ranol sulphate by the elimination of an OH group, together with the

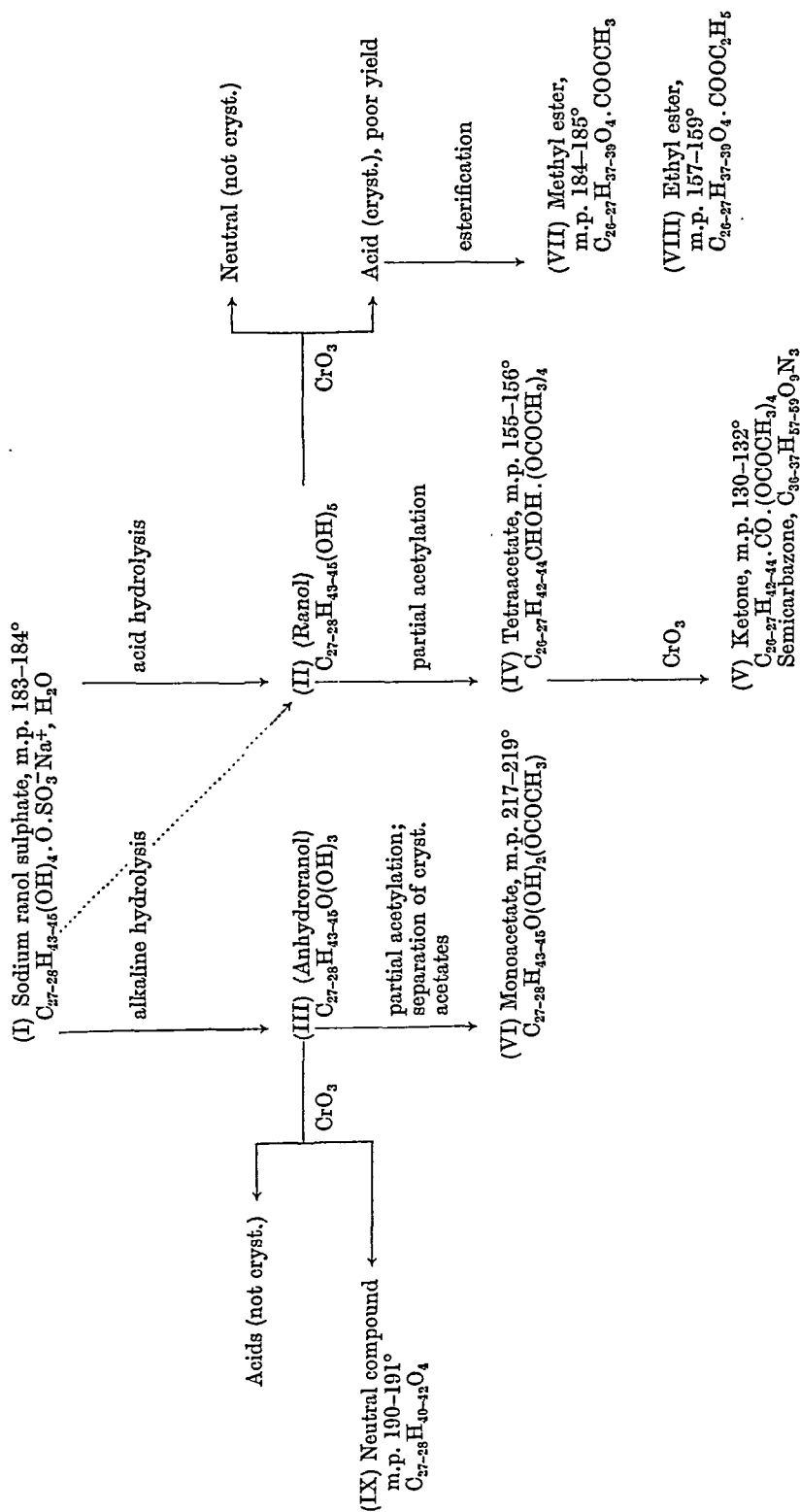
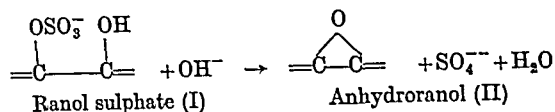


Fig. 1. Compounds derived from the bile of *Rana temporaria*. (Formulae on the assumption that ranol is a steroid whose molecule contains 27 or 28 carbon atoms.)

sulphate. Since the acetate (VI) appeared to be saturated, the chief product (not crystallized) formed by the above reaction most probably contained an oxide ring and is referred to tentatively as 'anhydroranol' (III), e.g.



The product of alkaline hydrolysis could also be converted by oxidation into a mixture from which was readily isolated a neutral substance (IX), whose probable formula can be explained on the assumption that a $-\text{CH}_2\text{OH}$ group in anhydroranol (III) was oxidized to $-\text{CHO}$ and two $=\text{CHOH}$ groups to $=\text{C}=\text{O}$, the oxide ring remaining unaffected.

A crystalline acid, purified as the methyl and ethyl esters (VII and VIII) was obtained by chromic oxidation of a hydrolysed mixture from which the only crystalline material isolated by acetylation was the tetraacetate (IV). It is tempting to assume that this acid arose by oxidation of a $-\text{CH}_2\text{OH}$ group in ranol to $-\text{COOH}$ and the conversion of four $=\text{CHOH}$ groups to $=\text{C}=\text{O}$, an interpretation which is supported by the analytical figures and which is shown in Fig. 1. However, the acid was obtained in very small yield and its preparation often failed, so that some doubt is felt as to its really arising in the manner suggested. The above esters were compared with, and found to be different from, the appropriate esters derived from dehydronor- and dehydrobismor-cholic acid, prepared from cholic acid as described by Shimizu & Kazuno (1936b).

EXPERIMENTAL

General. All melting points are uncorrected. Optical rotations were determined in a 1 dm. microtube; microanalyses were done by Weiler and Strauss, Oxford, and by Mr A. T. Macdonald, Edinburgh. Al_2O_3 was supplied by Hopkins and Williams Ltd.; l.p.=light petroleum, b.p. 40–60°. 20% CrO_3 was as described by Haslewood & Wootton (1951). H-test=Hammarsten's HCl test (Haslewood, 1943).

Substances derived from the bile of Rana temporaria

Sodium ranol sulphate (simplified preparation). A sample, m.p. 183–184°, was prepared and analysed by Haslewood & Wootton (1950). A simpler preparation was as follows: 392 gall bladders, preserved in ethanol, were ground in a mortar and thoroughly extracted with fresh ethanol. The filtered extract was evaporated and the residue extracted several times with ethanol in an evaporating dish at room temperature. The ethanol-insoluble residue was collected, washed with ethanol and dried *in vacuo* over H_2SO_4 . It was a light-brown microcrystalline powder of m.p. 185° (gas), and weighed 1.23 g. A further crop was obtained by evaporation of the ethanol extracts and washings, followed by re-

extraction of the residue with cold ethanol: the ethanol-insoluble material weighed 0.23 g. and had m.p. 182° (gas).

Acid hydrolysis of ranol sulphate. Sodium ranol sulphate (0.5 g.) was dissolved in 0.25N-HCl (20 ml.) and the solution in a flask was heated, with occasional shaking, in a gently boiling-water bath. After 1–2 hr. a gel formed and the amount of this slowly increased. After about 20 hr. heating the gel was collected on a filter, squeezed as far as possible free from aqueous acid, washed with water and dried *in vacuo* over H_2SO_4 . Yield, 0.35 g. (approx. 89%, calculated on the proposed formulæ) of a brown solid, giving a blue colour in the H-test. This is regarded as mainly crude ranol (II); it could not be crystallized.

Ranol tetraacetate (IV). The above material (0.1 g.) was dissolved in dry pyridine (1 ml.) with acetic anhydride (1 ml.) and the mixture left at about 20° for 19 hr. with occasional shaking. Dilution with aqueous HCl precipitated a brown gum which was collected after about 4 days and dissolved in ethanol. The filtered solution was evaporated and the residue crystallized from an approx. 30% (v/v) mixture of ether/l.p. The crude acetate (80 mg.) was then recrystallized from l.p./benzene from which it gave white needles (47 mg.) of m.p. 146–148°. Crystallization from dilute methanol and elution from Al_2O_3 with benzene, followed by further recrystallizations from l.p./benzene finally gave white needles of ranol tetraacetate (IV) which had m.p. 155–156°. $[\alpha]_D^{25} = -12 \pm 4^\circ$ in CHCl_3 (c, 1.0). (Found: C, 68.0, 67.8; H, 9.4, 9.4. $\text{C}_{35-36}\text{H}_{56-55}\text{O}_9$ requires C, 67.7–68.1; H, 9.0–9.2%.)

A sample of this acetate (15 mg.) was made from the product (24 mg.) of the acid hydrolysis of the purified bile salt (50 mg.) described by Haslewood & Wootton (1950).

Monoketone (V). The above acetate (47 mg.) in acetic acid (0.5 ml.) was treated with 20% CrO_3 (0.05 ml.), together with water (1 drop), to dissolve the precipitate. After 10 min. the solution was diluted and the product which separated on standing collected, washed with water and dissolved in ethanol. Evaporation of the ethanol left a residue which crystallized from l.p. and the l.p./benzene in white needles. This ketone (V) had m.p. 130–132°. (Found: C, 67.8; H, 8.2. $\text{C}_{35-36}\text{H}_{54-55}\text{O}_9$ requires C, 68.0–68.4; H, 8.7–8.9%.) $[\alpha]_D^{25} = +27 \pm 2^\circ$ in CHCl_3 (c, 0.5.) The semicarbazone, prepared in the usual way, was a partially crystalline powder of m.p. 171–175°. (Found: N, 6.1. $\text{C}_{36-37}\text{H}_{57-59}\text{O}_9\text{N}_3$ requires N, 6.2–6.1%.)

Esters (VII) and (VIII). The only samples of the acid giving these esters were prepared from a product made by alkaline hydrolysis of the bile salts under conditions not very accurately defined, but which included heating crude bile salts (c, 5 g.) for about 21 hr. in NaOH solution (30 g./l.). The neutral product which separated was collected, washed and dried; it gave on partial acetylation the acetate (IV) as the sole crystalline material isolated. A similar alkali-hydrolysed product could not be prepared again, under more carefully defined conditions of hydrolysis, for all other neutral substances obtained from the bile salts by alkali treatment gave derivatives apparently of the anhydroranol (see below). Several oxidations of the above product were carried out; the following experiment was one of those which led to crystalline material: the above-mentioned alkali-hydrolysed product (0.2 g.) in acetic acid (2 ml.) was treated, with cooling to about 20°, with 20% CrO_3 (2 ml.) added gradually with shaking. After 2 hr. the solution was diluted and treated with NaCl (excess). The gummy precipitate was collected after about 16 hr.; it was washed with water and

then boiled with N -NaOH. The cooled mixture was filtered from gummy neutral material and the filtrate acidified with H_2SO_4 . After the addition of NaCl (excess) the precipitated acid was collected, washed with water and dried *in vacuo* over H_2SO_4 . Yield: 80 mg. of acidic material which, from dilute ethanol, gave needles (25 mg.) of m.p. approx. 211–218° (decomp.). These were gently boiled for 1 hr. under reflux with 2.5 ml. of a mixture of ethanol (5 ml.) and H_2SO_4 (0.5 ml.). The cooled solution was diluted with Na_2CO_3 solution (excess) and the insoluble material collected, washed and crystallized from dilute ethanol, from which the *ethyl ester* (VIII) formed leaflets (10 mg.) of m.p. 157–159° (Found: C, 71.6, H, 8.7. $C_{29-30}H_{42-44}O_6$ requires C, 71.6–72.0; H, 8.6–8.8%.)

Another sample (25 mg.) of the above crude acid was esterified with diazomethane. The product, in ether, was washed with dilute NH_3 solution and water, and the solvent was removed. The residue, in benzene, was eluted from an Al_2O_3 column with benzene and crystallized from l.p./benzene, from which the *methyl ester* (VII) gave short white needles of m.p. 184–185° (Found: C, 71.5; H, 8.1. $C_{28-29}H_{40-42}O_6$ requires C, 71.2–71.6; H, 8.5–8.6%.)

Alkaline hydrolysis of ranol sulphate (leading to anhydro-ranol derivatives). Except in the case given above, it did not prove possible to prepare the same derivatives from the alkali-hydrolysed bile salts as could be made from the material derived by acid. An effective method of alkaline hydrolysis was as follows: a solution of the bile salts (0.2 g., purified and of m.p. 183–184°) in water (4 ml.) with 5*N*-NaOH (1 ml.) was sealed in a metal bomb and heated at about 110° for 8 hr. The bomb was cooled and its contents washed out with water. The precipitated solid was collected, washed with water and dried *in vacuo* over H_2SO_4 . Yield: 0.14 g. (approx. 92%, calculated on the suggested formulae) of a light brown powder, giving a feeble response in the H-test. By addition of $BaCl_2$ to the acidified liquors, it was found that the sulphate content (as S) of the bile salts was about 5.9%.

The acetate (VI). The above product (0.13 g.) in pyridine (1 ml.) was treated with acetic anhydride (1 ml.) and the mixture was warmed to about 50° and then left at about 22° for 16 hr. The solution was diluted with water and 5*N*-HCl and extracted with ether. The ether was washed with water, NH_3 solution, water, dried (Na_2SO_4) and evaporated. The residue readily crystallized from l.p./ether, giving crystals (70 mg.) of m.p. 175–204°. This material was recrystallized from dilute ethanol after which the product (40 mg.) had m.p. 202–216°; this mixture was then eluted from a column containing Al_2O_3 (0.5 g.) with benzene (40 ml.). After three recrystallizations of the eluted material (30 mg.) from dilute ethanol, the *acetate* (VI) formed glistening white leaflets of m.p. 217–219° (Found: C, 72.9, 73.3; H, 9.6, 9.7. $C_{29-30}H_{43-50}O_5$ requires C, 73.1–73.5; H, 10.1–10.2%.)

Alkaline hydrolysis of this acetate in the usual way gave a gelatinous solid which, after drying, responded feebly, if at all, to the H-test. On the other hand, the acetate (IV) (see above) gave on hydrolysis a gel, which, after drying, gave a definite purplish colour in this test.

The neutral compound (IX). The above-described material (0.1 g.), made by bomb hydrolysis with alkali, was dissolved in acetic acid (1 ml.) and treated at 19° with 20% CrO_3 (1 ml.) added gradually with mixing. After 1.2 hr. at 20°, the solution was diluted and saturated with NaCl. The precipitated solid was collected, washed with water and stirred with warm 0.1*N*-NaOH. The insoluble material was

filtered off. (The filtrate gave an amorphous acidic mixture (19 mg.) on acidification.) The alkali-insoluble precipitate was dissolved in ethanol and the filtered solution evaporated. The residue (23 mg.) was a partially crystalline solid of m.p. 173–179°. This was eluted from Al_2O_3 (0.4 g.) with benzene (15 ml.) and recrystallized from ether, from which it formed white needles. Thus prepared, the *neutral compound* (IX) had m.p. 189–190.5° (Found: C, 76.4, 76.6; H, 9.2, 9.1. $C_{27-28}H_{40-42}O_4$ requires C, 75.7–76.0; H, 9.4–9.5%.)

Derivatives of nor- and bisnor-cholic acid

Nor- and bisnor-cholic acids were prepared as described by Shimizu & Kazuno (1936b). Prepared in the usual way, *ethyl dehydronorcholate* crystallized from dilute ethanol as long white needles of m.p. 234–235° (decomp.). (Found: C, 71.4, 71.4; H, 9.0, 8.8. $C_{25}H_{36}O_5$ requires C, 72.1; H, 8.7%.)

Bisnorcholic acid was esterified with diazomethane and the product oxidized with CrO_3 in the usual way. The crude dehydro ester was purified by elution from Al_2O_3 with benzene and crystallized from l.p./benzene, from which *methyl dehydrobisnorcholate* formed long white needles of m.p. 191–193°, depressed by the methyl ester (VII). (Found: C, 70.9; H, 8.4. $C_{23}H_{32}O_5$ requires C, 71.1; H, 8.3%.)

DISCUSSION

Biological. The reported work agrees with that of the Japanese on *R. catesbiana* and on the Japanese toad, in that the main bile salt of *R. temporaria* also appears to be a neutral alcoholic substance or substances conjugated with sulphate. The amount of bile used in this research was insufficient for the identification of the minor quantities of acids which were also present.

The 'sodium ranol sulphate' now investigated appears to resemble closely, if not to be identical with, the sulphate, m.p. 178°, isolated by Kuraiti & Kazuno (1939) from *R. catesbiana*, but a careful scrutiny of Kazuno's (1940) report on pentahydroxy-bufostane and other neutral substances from toad bile has failed to suggest the identity of these or any of their derivatives with material from *R. temporaria*. The conclusion is that at least two species of *Rana* probably contain the same bile salt, but that this is different from similar compounds in the bile of one species of *Bufo*.

Okasaki (1944) has reported that the chief bile salt of the aquatic salamander *Diemyctylus phyllorhogaster* is likewise the sulphate ester of a neutral steroid alcohol.

The presence of such neutral substances in bile of amphibia would suggest, on the hypotheses put forward by Haslewood & Wootton (1950), that these creatures are evolutionarily of a primitive type and that they agree with at least some of the elasmobranch fishes and with the carp *Cyprinus carpio* (Haslewood, 1951) in this respect. It would be unwise at present to suggest that any closer biological

relationship is indicated by the similarities so far revealed in the chemical nature of the neutral bile salts.

Chemical. The scheme put forward in Fig. 1 to explain the relationships between the crystalline substances isolated must be regarded as tentative. An alternative explanation, that 'sodium ranol sulphate' is a mixture, components of which give rise to the ranol and anhydroranol derivatives, can hardly be upheld since the acetates IV and VI were isolated exclusively after acid and alkaline hydrolysis, respectively, from the same purified sample of the sodium salt. Moreover, although it is difficult to be certain on this point, purified sodium ranol sulphate behaved as a single reproducible compound in melting point and general properties; its analysis agreed fairly well with the postulated formulae. However, the case for the scheme in Fig. 1 would be greatly strengthened if ranol and anhydroranol themselves could be obtained in crystalline form. No trace was detected of the crystalline unsaturated 'trihydroxychole' of Kurauti & Kazuno (1939), which was presumably derived from their sulphate by elimination of the sulphonyl group, together with an adjacent hydrogen atom, during alkaline hydrolysis.

The amount of significance which should be attached to the feeble or negative response to the Hammarsten test given by anhydroranol is doubtful, for it was found that the carbinols derived by reaction of the Grignard reagent with methyl cholate and methyl norcholate also responded feebly to this test. Clearly, response to a Hammarsten test can be greatly modified by the side chain of the steroid, and although a positive reaction is very probably, in a natural steroid, indicative of hydroxyl groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$, a failure to give a blue or purple colour cannot necessarily be taken as indicating the absence of such groups (see also, for example, Shimizu & Kazuno, 1936*a, b*). The colour given by ranol certainly suggests that this substance has three of its hydroxyl groups in the above-mentioned positions. One of the remaining two OH groups in ranol is, possibly, primary, and both were readily acetylated. If the structure suggested (see Results) for compound IX is correct, one at least of the nuclear OH groups must be involved in the

formation of the oxide ring in anhydroranol. The monoketone (V) appears to be derived from a steroid containing a free hydroxyl group at $C_{(12)}$ (compare pythocholic lactone, Haslewood & Wootton, 1951).

The analytical figures do not enable one to choose between formulae for a C_{27} or C_{28} steroid for ranol and its derivatives. If ranol is in fact a C_{28} steroid, it would be difficult to explain its derivation from cholesterol, an origin frequently suggested for scymnol and the C_{21} bile acids.

One of the chief objects of this work has been the development of methods which can be applied to other species and might then be expected to lead to the isolation of identifiable crystalline substances. Such a substance, which might be of value in future work, is the so-called ranol tetraacetate (IV).

SUMMARY

1. The chief bile salt found in the frog *Rana temporaria* was a sulphate, now temporarily named 'sodium ranol sulphate', of a pentahydric alcohol, 'ranol', probably $C_{27-28}H_{43-45}(OH)_5$.

Derivatives of this alcohol could be isolated after acid hydrolysis of the sulphate. After alkaline hydrolysis, however, the only crystalline compounds isolated appeared, except in one experiment, to be derived from an 'anhydroranol', a saturated substance whose molecular formula is less by H_2O than that of ranol. It is suggested that the molecule of anhydroranol, like that of scymnol, contains an oxide ring.

2. A relationship between the seven crystalline substances now obtained in purified form from frog's bile is tentatively suggested. It is thought that some of these substances may be of value in future investigations of bile from other species.

3. Ranol may be a steroid whose molecule contains a primary hydroxyl group, with secondary hydroxyl groups at $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$. The remaining OH group was readily acetylated.

4. The possible evolutionary significance of similar sulphate esters in the bile of vertebrates is briefly discussed.

The author thanks Mr D. H. Gadd for his kindness in collecting the frog's bile used in this work.

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Studies on the Analysis of Vitamins D

6. A NOTE ON THE PREPARATION OF FLORIDIN EARTH FOR VITAMIN D CHROMATOGRAPHY

By J. GREEN

Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

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Further work on the use of floridin earth for the chromatographic analysis of materials containing vitamin D, as described in earlier papers (Green, 1951*a-c*), has shown that the preparation of the earth before use is of a more critical nature than had originally been thought. The adsorbent used in these earlier papers was 'prepared for Emmerie's test' supplied by British Drug Houses Ltd. The preparation process consists essentially of an activation with boiling hydrochloric acid under controlled conditions; activation appears to take place first by the removal of carbonates and other soluble materials and, secondly, by the breakdown of the surface structure of the earth. Trials on recently received samples of earth show that this preliminary preparation can 'over-activate' the earth by carrying the breakdown process too far. If this extensive structural breakdown (the process is an irreversible one) should occur, the earth is then unsuitable for chromatography, destroying 20–40 % of the vitamin D during the adsorption procedure. The earth is, of course, still quite suitable for vitamin E chromatography.

Whilst it is hoped that suitably prepared samples of floridin may be available in the future, a satisfactory laboratory method of preparation has been found to be as follows: 100 g. of the crude untreated earth (obtainable from British Drug Houses Ltd.) is made into a slurry with 260 g. of concentrated hydrochloric acid and 40 g. of water. The mixture is boiled for 30 min. under reflux. After cooling, the floridin is washed with water, first by decantation and then on a filter at the pump, until free from acid. The floridin is then dried at 37–40° in thin layers, turning the layers frequently. When dry, the earth is passed through a 160-mesh sieve; the retained portion is suitable for use and remains so indefinitely.

Although the length of time for the activation as stated above appears to be satisfactory, it is possible that this may have to be varied slightly from batch to batch of untreated earth. It is therefore recommended that the prepared sample be tested for quantitative adsorption and elution.

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Polarization of the Fluorescence of Macromolecules

1. THEORY AND EXPERIMENTAL METHOD

By G. WEBER*

Biochemical Laboratory, University of Cambridge

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According to the theory developed by Perrin (1926), the partial polarization of the fluorescent light emitted by molecules in solution depends upon the relation of their rotational relaxation time to the lifetime of the excited state of the fluorescence.

The measurements of Gaviola (1927), Szymanowski (1935) and Dushinsky (1936) show that the lifetime of the excited state of the fluorescence is a quantity of the order of 10^{-8} sec. for many dyes in water solution. As the relaxation time of the rotation of these molecules in water is much shorter than 10^{-8} sec., almost completely depolarized radiation is to be expected and is in fact observed. On the other hand, fluorescent macromolecules, e.g. proteins, should already emit partially polarized fluorescence in water solution as their relaxation times are of the required order of magnitude. Measurements of the degree of polarization of the fluorescence should in consequence afford a convenient means of determining the relaxation times of macromolecules in dilute solution under a variety of circumstances.

In this paper are described the general principles which attend the application of this method to the study of macromolecules. The following paper of this series (Weber, 1952) describes experiments done with proteins rendered fluorescent by the conjugation with a small fluorescent molecule.

THEORY

Perrin (1926, 1936) has developed the theory of the polarization of the fluorescence of solutions on the assumption that the emitting molecules carry rigidly bound linear oscillators of absorption and emission and that the molecular rotations are described by Einstein's (1906) equation. The first assumption can be considered experimentally proved (Feofilov, 1943). Although the classical experiments of J. Perrin (1909) have shown that the rotation of large, visible particles follows the Einstein equation, some doubt may be entertained as to its validity in the case of dissolved molecules which do not greatly differ in size from the molecules of the solvent. The protein molecules fall midway between these two extremes, and the measurements

of their relaxation times by the dielectric dispersion method (Onley, 1942) indicate that the equation of Einstein cannot seriously be at fault. However, the fact that the real shape of the particles is not known and that the measurements have been carried out over a limited range of temperatures and viscosities does not allow a decision as to how closely the theory is followed.

Let us consider a system of co-ordinates $O(xyz)$. The fluorescent solution is placed at O in a square cell with faces oriented normally to the co-ordinate axes. The exciting light travels in the xO direction, and unless otherwise stated we shall assume it to be completely polarized with its electric vector in the Oz direction. The fluorescent light emitted in the Oy direction, i.e. at right angles both to the direction of propagation of the exciting light and to the direction of the electric vector is observed. Its partial polarization p is defined by the equation

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}, \quad (1)$$

where I_{\parallel} is the component of the intensity emitted in the direction Oy with its direction of vibration parallel to that of the exciting light, while I_{\perp} is the component normal to the former, i.e. in the Ox direction.

According to Perrin (1926) the value of p for a spherical molecule is given by

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau_0 \right), \quad (2)$$

where R is the gas constant, T the absolute temperature, η the viscosity of the solvent, V the molecular volume of the fluorescent molecule and τ_0 the lifetime of the excited state of the fluorescence; p_0 is clearly the value of p when $T/\eta \rightarrow 0$, i.e. when no depolarization by molecular rotation takes place. In practice it differs little from the polarization observed in a medium of high viscosity like glycerol. According to the above equation, if $1/p$ is plotted against T/η a straight line is obtained cutting the $1/p$ axis at $1/p_0$. Experimentally, for fluorescent dyes with $V \approx 500$ in glycerol-water mixtures it is found that the relation just mentioned obtains down to viscosities of 10–15 centipoises, but for

* Beit Memorial Fellow.

viscosities below these values the polarization is lower than predicted (Perrin, 1926; Wawilow, 1936). Several explanations have been advanced, notably that Einstein's law is not valid in these conditions (Perrin, 1936). Whatever the explanation, if a change in molecular volume has the same effect as a corresponding change in viscosity, Perrin's law should hold accurately for molecules of $V > 10^4$ in media of viscosity of 1 centipoise.

Simultaneous excitation of several oscillators

If several oscillators corresponding to one or more molecular species in solution are simultaneously excited, the observed polarization \bar{p} is related to the individual polarizations p_i that would obtain if each type of oscillator were the only one excited, in the following manner:

$$\text{Setting} \quad I_{\parallel} + I_{\perp} = F_i,$$

$$p_i = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{I_{\parallel} - I_{\perp}}{F_i}.$$

$$\text{Moreover} \quad I_{\parallel} = \sum_i I_{\parallel i} \quad \text{and} \quad I_{\perp} = \sum_i I_{\perp i}.$$

$$\text{Therefore} \quad \bar{p} \equiv \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{\sum_i F_i p_i}{\sum_i F_i}. \quad (3)$$

Addition law of the polarizations of several oscillators

The total intensity of the radiation emitted by a point of the fluorescent source can be represented by three orthogonal components. Choosing for the directions of the components the co-ordinate axes, we have in the case of non-polarized radiation,

$$I_{\parallel} = I_{\perp} = i,$$

the total intensity being proportional to $3i$. If the fluorescence excited by polarized light as described, becomes now partially polarized to the extent p_i , while the total emitted intensity remains constant, Curie's law of symmetry (Perrin, 1929) shows that the total radiation is then proportional to

$$I_{\parallel} + 2I_{\perp} = 3i.$$

This requires

$$I_{\parallel} = i + \Delta, \quad (4)$$

$$I_{\perp} = i - \frac{1}{2}\Delta. \quad (5)$$

From the last equations, 4 and 5, together with 1, we have for the i th oscillator species in solution:

$$\frac{\Delta_i}{2} = \frac{2i_i}{3 \left(\frac{1}{p_i} - \frac{1}{3} \right)},$$

$$F_i p_i = \frac{2i_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)}.$$

Defining $f_i = \frac{i_i}{\sum_i i_i}$ as the contribution of the i th oscillator species to the total fluorescent intensity, the last equation becomes

$$F_i p_i = 2 \left(\sum_i i_i \right) \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}}.$$

On the other hand, from Eqn. 3,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{\sum_i F_i}{\sum_i F_i p_i} - \frac{1}{3} = \frac{\sum_i F_i p_i \left(\frac{1}{p_i} - \frac{1}{3} \right)}{\sum_i F_i p_i}.$$

Introducing in the latter the value of $F_i p_i$ just derived,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}}}. \quad (6)$$

Thus $1/\bar{p} - \frac{1}{3}$ is the harmonic mean of the quantities $1/p_i - \frac{1}{3}$ weighed according to the contribution to the total fluorescent intensity of the solution.

Eqns. 4 and 5 refer to excitation with polarized light. In this case the electric vector of the exciting light is an axis of symmetry and $3i = I_{\parallel} + 2I_{\perp}$. If the fluorescence is excited with natural light the direction of propagation is now an axis of symmetry (Perrin, 1929) and the total radiation of the source is proportional to $3i = 2I_{\parallel} + I_{\perp}$. Eqns. 4 and 5 become now respectively,

$$I_{\parallel} = i_i + \frac{1}{2}\Delta_i; \quad I_{\perp} = i_i - \Delta_i, \quad (7)$$

and consequently

$$\frac{1}{\bar{p}_n} + \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\frac{1}{p_{in}} + \frac{1}{3}}}, \quad (8)$$

where the subscript n refers to the same quantities as before but on excitation with natural light. Perrin's law for the excitation with natural light reads

$$\frac{1}{p_n} + \frac{1}{3} = \left(\frac{1}{p_{0n}} + \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau_0 \right). \quad (9)$$

Therefore in the equations to follow it will only be necessary to write $1/p_n + \frac{1}{3}$ and $1/p_{0n} + \frac{1}{3}$ instead of $1/p - \frac{1}{3}$ and $1/p_0 - \frac{1}{3}$ respectively, to pass from the case of excitation with polarized light to that of excitation with natural light.

If $1/\bar{p} - \frac{1}{3}$ is plotted against T/η in the case of a system of several components the slope, obtained by differentiating Eqn. 6, is

$$\left. \begin{aligned} \frac{d}{d(T/\eta)} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) &= \sum_i \frac{d \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d \left(\frac{1}{p_i} - \frac{1}{3} \right)} \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)}, \\ \frac{d \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d \left(\frac{1}{p_i} - \frac{1}{3} \right)} &= \frac{\frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2}}{\left(\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right)^2}. \end{aligned} \right\} \quad (10)$$

If the polarization of the fluorescence given by the i th oscillator species when alone in solution is described by Perrin's equation,

$$\left. \begin{aligned} \frac{1}{p_i} - \frac{1}{3} &= \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \left(1 + \frac{3\tau_i}{\rho_i} \right), \quad \rho_i = \frac{3\eta V_i}{RT}, \\ \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)} &= \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \frac{3\tau_i}{\rho_i} \frac{d \left(\frac{1}{T/\eta} \right)}{d(T/\eta)}, \end{aligned} \right\} \quad (11)$$

introducing the values of the partial differentials into Eqn. 10 and performing the additions,

$$\frac{d}{d(T/\eta)} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{\sum_i \frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2} \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \frac{3\tau_i}{\rho_i}}{\frac{T}{\eta} \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right]^2}. \quad (12)$$

Similarly, we may calculate

$$\frac{d^2}{d(T/\eta)^2} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{-2 \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \left(\frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d \left(\frac{1}{T/\eta} \right)} \right)^2 \right] \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right] + 2 \left[\sum_i \frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2} \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d \left(\frac{1}{T/\eta} \right)} \right] \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right]}{\left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right]^3}.$$

Replacing $\frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)}$ by its value given in Eqn. 11 and performing the multiplication of the series,

$$\frac{d^2 \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d(T/\eta)^2} = \frac{- \sum_i \frac{f_i f_j}{\left(\frac{1}{p_i} - \frac{1}{3} \right) \left(\frac{1}{p_j} - \frac{1}{3} \right)} \left(\frac{3\tau_i/\rho_i}{1 + 3\tau_i/\rho_i} - \frac{3\tau_j/\rho_j}{1 + 3\tau_j/\rho_j} \right)^2}{\left(\frac{T}{\eta} \right)^2 \left(\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right)^3} \quad (i \neq j). \quad (12a)$$

Therefore the first derivative is always positive and the second either zero or negative and the plotting of $1/\bar{p}$ against T/η is either a straight line or a curve concave towards the T/η axis. Eqn. 11 shows also that if the oscillators differ only in the value of the limiting polarization, p_{0i} , no curvature is to be observed. It is in fact easy to deduce that in such case the observed polarization is given by the equation

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\left(\sum_i \frac{f_i}{\frac{1}{p_{0i}} - \frac{1}{3}} \right)} \left(1 + \frac{\tau_0}{\rho} \right). \quad (12b)$$

It is found experimentally that p_0 varies with the wavelength of the exciting light. Eqn. 12b shows, however, that the calculated value of the relaxation time of the rotation will be the same whether the light used for the excitation is monochromatic or not. This has been experimentally demonstrated by Perrin (1929). Eqn. 12b also shows that if the excitation is due to more than one wavelength a

change in the observed polarization may in certain cases be due to a change in the absorption spectrum of the fluorescent solution leading to a different value of \bar{p}_0 , and not to a change in the lifetime of the excited state or the rotational relaxation time.

*Molecules of different size carrying
the same fluorescent oscillator*

There is in this case only one lifetime of the excited state and one limiting polarization, i.e. $p_{0i} = p_0$ and $\tau_i = \tau_0$. Eqn. 6 can be written in the form

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau_0}{\rho_i} \right)}} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}.$$

Adding to both members of the last equation the quantity

$$\left(\frac{1}{p_0} - \frac{1}{3} \right) \left\{ 1 + 3\tau_0 \sum_i \frac{f_i}{\rho_i} - \sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) \right\} = 0,$$

and rearranging the right-hand side

$$\frac{1}{\bar{p}} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left\{ 1 + 3\tau_0 \sum_i \frac{f_i}{\rho_i} - \left[\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) - \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}} \right] \right\}. \quad (13)$$

At values of T/η , such that all values of $3\tau_0/\rho_i$ are small compared to unity, the term between the square brackets is negligible. Therefore the initial slope in the plotting of $1/\bar{p}$ against T/η is proportional to the harmonic mean of the relaxation times of the particles weighted according to their contribution to the total emitted intensity. At values of T/η such that the term between the square brackets becomes significant, the value of $1/\bar{p} - \frac{1}{3}$ is always smaller than that corresponding to the initial slope, and the relative decrease is, according to Eqn. 13,

$$\frac{\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{\frac{1}{\bar{p}} - \frac{1}{3}} = \frac{\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) - \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}}{\frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}} = \left(\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) \right) \left(\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}} \right) - 1.$$

Writing

$$1 = \left(\sum_i f_i \right) \left(\sum_i f_i \right) = \sum_i f_i^2 + 2 \sum_{i \neq j} f_i f_j$$

and performing the multiplication of the series,

$$\frac{\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{\frac{1}{\bar{p}} - \frac{1}{3}} = \sum_{i \neq j} \frac{f_i f_j 9\tau_0^2 \left(\frac{1}{\rho_i} - \frac{1}{\rho_j} \right)^2}{\left(1 + \frac{3\tau_0}{\rho_i} \right) \left(1 + \frac{3\tau_0}{\rho_j} \right)} = \sum_{i \neq j} \frac{f_i f_j}{\left(\frac{1 + 3\tau_0/\rho_j}{3\tau_0/\rho_j} \right)^2 \left(\frac{1}{n-1} \right)^2 + \left(\frac{1 + 3\tau_0/\rho_j}{3\tau_0/\rho_j} \right) \left(\frac{1}{n-1} \right)}, \quad (14)$$

in which

$$n = \frac{\rho_j}{\rho_i}, \quad n \geq 1.$$

In the case of only two molecular species in solution the last equation reads

$$\frac{\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{\frac{1}{\bar{p}} - \frac{1}{3}} = \frac{f_1 f_2}{\left(\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} \right)^2 \left(\frac{1}{n-1} \right)^2 + \left(\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} \right) \left(\frac{1}{n-1} \right)}. \quad (15)$$

From Eqn. 2

$$\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} = \frac{\frac{1}{p_1} - \frac{1}{3}}{\frac{1}{p_1} - \frac{1}{p_0}}$$

Substituting this in Eqn. 15 we have

$$\delta\left(\frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}}\right) = \frac{f_1 f_2}{\left(\frac{\frac{1}{p_1} - \frac{1}{3}}{\frac{1}{p_1} - \frac{1}{p_0}}\right)^2 \left(\frac{1}{n-1}\right)^2 + \left(\frac{\frac{1}{p_1} - \frac{1}{3}}{\frac{1}{p_1} - \frac{1}{p_0}}\right) \left(\frac{1}{n-1}\right)}$$

The presence of experimentally significant curvature requires that the lowest polarizations attained depart from the values corresponding to the initial tangent by an amount

$$\frac{\delta\left(\frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}}\right)}{\frac{1}{\bar{p}} - \frac{1}{3}} \geq 2.5\epsilon,$$

where ϵ is the standard error of the observations. If we assume $\epsilon = 0.02$ and $1/p_1 < 10$, as in any case $1/p_0 \geq 2$, the condition for observable curvature becomes

$$\frac{f_1 f_2}{\frac{25}{16} \left(\frac{1}{n-1}\right)^2 + \frac{5}{4} \left(\frac{1}{n-1}\right)} \geq 0.05,$$

n is a minimum if $f_1 = f_2 = \frac{1}{2}$, and therefore $n > \frac{3}{2}$. Thus in a solution of two spherical molecules a deviation from the linear law will only be present under the experimental conditions described if the ratio of the relaxation times of the rotation is greater than $\frac{3}{2}$, and conversely if one molecular species carry two different oscillators in equal amounts a deviation from the linear law requires a similar ratio for the lifetimes of the excited state.

Effect of the presence of a fluorescent impurity of low molecular weight

In the case of two spherical molecules of relaxation times ρ_1 and ρ_2 Eqn. 13 becomes

$$\frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = 1 + \left[\frac{3\tau_0}{\rho_2} (f_1 q + f_2) \frac{\left(1 + \frac{3\tau_0}{\rho_2} \left(\frac{q}{f_1 q + f_2}\right)\right)}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)} \right], \quad (16)$$

with $q = \rho_2/\rho_1$, $q \leq 1$. The slope S on the plotting of $\frac{1/\bar{p} - 1/3}{1/p_0 - 1/3}$ against T/η can be obtained by differentiating Eqn. 16:

$$S = \frac{(f_1 q + f_2) \frac{3\tau_0}{\rho_2} + 2q \left(\frac{3\tau_0}{\rho_2}\right)^2 + q(f_1 + f_2 q) \left(\frac{3\tau_0}{\rho_2}\right)^3}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)^2 \left(\frac{T}{\eta}\right)}. \quad (17)$$

The tangent to the curve at a given value of T/η cuts the ordinate axis at the value $1/p'_0$ defined by the equation

$$\frac{\frac{1}{p'_0} - \frac{1}{p_0}}{\frac{1}{p_0} - \frac{1}{3}} = \frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} - S \frac{T}{\eta} = \frac{\left(\frac{3\tau_0}{\rho_2}\right)^2 (1-q)^2 f_1 f_2}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)^2}. \quad (18)$$

If $q \ll 1$ and $3\tau_0/\rho_2 \gg 1$ we have the case in which a macromolecule and a low molecular weight impurity coexist in solution. Eqn. 18 shows that then a very simple relation obtains approximately between the relative increase in the intercept and the contribution of the impurity to the total fluorescent emission, namely:

$$f_2 \approx \frac{\frac{1}{p'_0} - \frac{1}{p_0}}{\frac{1}{p_0} - \frac{1}{3}}. \quad (19)$$

Moreover, if

$$\frac{3\tau_0}{\rho_2} f_1 \gg 1, \quad (20)$$

$$\frac{\frac{3\tau_0}{\rho_2} (f_1 q + f_2)}{1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)} \approx \frac{f_2}{f_1},$$

and the right-hand side of Eqn. 16 reduces to

$$\frac{1}{f_1} \left(1 + \frac{3\tau_0}{\rho_1}\right).$$

From the last, and Eqn. 19, we have

$$\left(\frac{1}{\bar{p}} - \frac{1}{3}\right) \approx \left(\frac{1}{p'_0} - \frac{1}{3}\right) \left(1 + \frac{3\tau_0}{\rho_1}\right). \quad (21)$$

Eqn. 21 has the same form as 2, p'_0 taking the place of p_0 . Therefore the linear law is followed over the range of values of T/η such that $(3\tau_0/\rho_2)f_1 \gg 1$ and the relaxation time calculated from the slope and intercept is the true relaxation time of the rotation of the macromolecule.

Polarization of the fluorescence emitted by ellipsoidal molecules carrying randomly oriented oscillators

Perrin (1936) has developed the theory of the polarization of the fluorescence emitted by molecules of any shape. There appears to be no simple theory for ellipsoids of revolution. The value of p is in that case an explicit function not only of the principal relaxation times of the rotation, but also

of the three angles determined by the oscillators of absorption and emission of light and the axis of revolution of the ellipsoid.

The general equation (Perrin, 1929),

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{3 \overline{\cos^2 \omega} - 1}, \quad (22)$$

where $\overline{\cos^2 \omega}$ is the average cosine square of the angle swept by the emission oscillator between the times of absorption and emission of light, may, however, be applied in certain cases. If OA (Fig. 1) denotes the

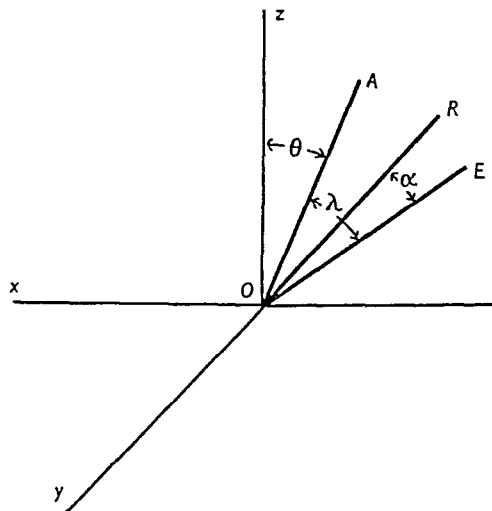


Fig. 1. Explanation in the text.

direction of the oscillator of absorption, OE the direction of the emission oscillator and OR the major axis of the ellipsoid, for the last equation to be valid it is necessary that $\overline{\cos^2 \omega}$ be independent of azimuth about OE . This requires all positions of the ellipsoid with OE in a particular direction to have equal weight. The weight of each position is given by $\cos^2 \theta$, where θ is the angle between the absorption oscillator and the electric vector of the exciting light Oz . The condition that all positions of the molecule obtained by rotation about OE have equal weight can only be fulfilled in two particular instances:

(a) OA and OE are coincident;

(b) OA and OE make a fixed angle λ between them, but for a given direction of OE and OR all azimuths of OA about OE are equally probable. Clearly this is impossible if the axis of revolution of the molecule makes fixed angles with the oscillators, but it will represent the actual case when a small fluorescent molecule is attached to a much larger non-fluorescent one by adsorption or covalent bonding, provided there is no particular orientation of the molecules with respect to each other. If the larger molecule is an ellipsoid of revolution, $\overline{\cos^2 \omega}$

will be a function of the angle α between OR and OE and the two principal relaxation times of the rotation of the ellipsoid. Moreover, it will be necessary to sum over all values of α since random orientation of the second molecule with respect to the first has been assumed. By application of Eqn. 6 we then have

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{6 \int_0^{\frac{1}{2}\pi} \overline{\cos^2 \omega}(\alpha) d\alpha - 1}, \quad (23)$$

$$\text{where } \overline{\cos^2 \omega}(\alpha) = \frac{1}{\tau_0} \int_0^\infty \overline{\cos^2 \omega}(\alpha, t) e^{-t/\tau_0} dt. \quad (24)$$

Calculation of $\overline{\cos^2 \omega}$. Consider two positions of the ellipsoid; in the first the principal axes of the ellipsoid are coincident with the co-ordinate axes and the components of the oscillators of emission along the co-ordinates are $\cos \alpha$, $\cos \beta$ and $\cos \gamma$ respectively. After a rotation about the centre the components are $\cos \alpha'$, $\cos \beta'$ and $\cos \gamma'$. Therefore ω , the angle determined by the two directions of the oscillator of emission, is defined by,

$$\cos \omega = \cos \alpha \cos \alpha' + \cos \beta \cos \beta' + \cos \gamma \cos \gamma'.$$

If \cos_{ij} ($i = 1, 2, 3$; $j = 1, 2, 3$) denotes the angle between the direction of a principal axis i of the ellipsoid in its second position and an axis j in the first,

$$\begin{aligned} \cos^2 \omega = & [\cos^2 \alpha \cos_{11} + \cos \alpha \cos \beta \cos_{21} \\ & + \cos \alpha \cos \gamma \cos_{31} + \cos \alpha \cos \beta \cos_{12} \\ & + \cos^2 \beta \cos_{22} + \cos \beta \cos \gamma \cos_{32} \\ & + \cos \alpha \cos \gamma \cos_{13} + \cos \beta \cos \gamma \cos_{23} \\ & + \cos^2 \gamma \cos_{33}]^2. \end{aligned}$$

Taking into account that the mean value of the double products of the form $\overline{\cos_{ij} \cos_{jk}}$, where one or more indices appear only once are null by symmetry (Perrin, 1936), and setting

$$\cos^2 \alpha = z, \quad \cos^2 \beta = \cos^2 \gamma = \frac{1}{2}(1 - z),$$

we have

$$\begin{aligned} \overline{\cos^2 \omega} = & z^2 \overline{\cos_{11}^2} + \frac{(1-z)^2}{4} (\overline{\cos_{22}^2} + \overline{\cos_{33}^2}) \\ & + z(1-z) (\overline{\cos_{31}^2} + \overline{\cos_{31}^2} + \overline{\cos_{11} \cos_{22}} \\ & + \overline{\cos_{12} \cos_{21}} + \overline{\cos_{11} \cos_{33}} + \overline{\cos_{13} \cos_{31}} \\ & + \frac{(1-z)^2}{2} (\overline{\cos_{32}^2} + \overline{\cos_{22} \cos_{33}} + \overline{\cos_{23} \cos_{32}}), \end{aligned} \quad (25)$$

where the bar denotes average value.

To obtain $\overline{\cos^2 \omega}(\alpha, t)$ a function of the time t , we introduce the values calculated by Perrin (1936) for the average cosine square and double products of the angles determined by the axes of the ellipsoid of

revolution submitted to brownian movement. These are

$$\left. \begin{aligned} \overline{\cos^2_{11}} &= \frac{1}{3} + \frac{2}{3} e^{-3t/\rho_1}, \\ \overline{\cos^2_{22}} = \overline{\cos^2_{33}} &= \frac{1}{3} + \frac{1}{6} e^{-3t/\rho_1} + \frac{1}{2} \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \\ \overline{\cos^2_{12}} = \overline{\cos^2_{21}} = \overline{\cos^2_{13}} = \overline{\cos^2_{31}} &= \frac{1}{3} - \frac{2}{3} e^{-3t/\rho_1}, \\ \overline{\cos^2_{23}} = \overline{\cos^2_{32}} &= \frac{1}{3} + \frac{1}{6} e^{-3t/\rho_1} - \frac{1}{2} \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \\ \overline{\cos_{22} \cos_{11}} + \overline{\cos_{12} \cos_{21}} &= \overline{\cos_{33} \cos_{11}} + \overline{\cos_{13} \cos_{31}} \\ &= \exp \left[- \left(\frac{2}{\rho_1} + \frac{1}{\rho_2} \right) t \right], \\ \overline{\cos_{22} \cos_{33}} + \overline{\cos_{23} \cos_{32}} &= \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \end{aligned} \right\} \quad (26)$$

ρ_1 and ρ_2 are the relaxation times of the rotation of the ellipsoid about the long and short axis respectively. By introduction of these values in Eqn. 25 and integration of Eqn. 24:

$$\overline{\overline{\cos^2 \omega(\alpha)}} = \frac{1}{3} + \frac{(1-3z)^2}{6} \frac{1}{\left(1 + \frac{3\tau_0}{\rho_1}\right)} + \frac{(1-z)^2}{2} \frac{1}{\left(1 + \left(\frac{4}{\rho_2} - \frac{1}{\rho_1}\right)\tau_0\right)} + 2z(1-z) \frac{1}{\left(1 + \left(\frac{2}{\rho_1} + \frac{1}{\rho_2}\right)\tau_0\right)}, \quad (27)$$

and finally integrating Eqn. 23 after introducing $\overline{\overline{\cos^2 \omega(\alpha)}}$

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{AB}{C} \left(\frac{8}{3 + 6 \frac{B}{C} - \frac{B^2}{C^2}} \right), \quad (28)$$

where

$$A = 1 + \frac{3\tau_0}{n_1\rho_0}, \quad B = 1 + \left(\frac{4}{n_2} - \frac{1}{n_1} \right) \frac{\tau_0}{\rho_0}, \quad C = 1 + \left(\frac{2}{n_1} + \frac{1}{n_2} \right) \frac{\tau_0}{\rho_0},$$

$$\frac{\rho_1}{\rho_0} = n_1, \quad \frac{\rho_2}{\rho_0} = n_2, \quad \rho_0 = \frac{3\eta V_e}{RT}, \quad V_e = \text{volume of the ellipsoid.}$$

The last equation can also be written

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{\left(1 + \frac{3\tau_0}{n_1\rho_0} \right) \left(1 + \left[\frac{4}{n_2} - \frac{1}{n_1} \right] \frac{\tau_0}{\rho_0} \right)}{1 + \frac{1}{2} \left(\frac{5}{n_2} + \frac{1}{n_1} \right) \frac{\tau_0}{\rho_0} + \frac{9}{8} \frac{\frac{\tau_0^2}{\rho_0^2} \left(\frac{1}{n_2} - \frac{1}{n_1} \right)^2}{\left(1 + \left[\frac{2}{n_1} + \frac{1}{n_2} \right] \frac{\tau_0}{\rho_0} \right)}}, \quad (29)$$

which clearly shows that on plotting $1/p - \frac{1}{3}$ against τ_0/ρ_0 or, which is equivalent, against T/η , the ratio of the initial slope to that of the sphere of the same volume is

$$\frac{S}{S_0} = \frac{1}{2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right), \quad (30)$$

the harmonic mean of the ratios of the relaxation times of the ellipsoid to the relaxation time of the sphere of the same volume. If the last term of the denominator of Eqn. 29 is neglected the equation describes within 1% the depolarization due to

molecules of any elongation up to $\tau_0/\rho_0 = 0.13$ and within 5% up to $\tau_0/\rho_0 = 0.5$. A better and more interesting approximation is given by the equation

$$\left. \begin{aligned} \frac{1}{p} - \frac{1}{3} &= \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau_0}{\rho_0 n_p} \left[\frac{1 + \frac{3\tau_0}{\rho_0 n_a}}{1 + \frac{3\tau_0}{\rho_0 n_p}} \right] \right), \\ \frac{1}{n_p} &= \frac{1}{2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right), \quad n_a = \frac{n_1 + n_2}{2}. \end{aligned} \right\} \quad (31)$$

The last is obtained immediately from Eqn. 6 in the case of two spherical molecules present in equal amounts ($f_1 = f_2 = \frac{1}{2}$) and having relaxation times ρ_1 and ρ_2 . The random distribution of the directions of the oscillators in an ellipsoidal molecule result in almost complete uncoupling of the effects of both relaxation times, so that qualitatively and almost quantitatively the situation is much the same as if both relaxation times belonged to different molecules.

Depolarization by flat and elongated molecules

If the molecule is a flat ellipsoid of revolution its two principal relaxation times never differ between themselves by more than 10% (Perrin, 1934). Consequently, on plotting $1/p$ against T/η the departure from linearity is negligible and a flat ellipsoidal molecule cannot be distinguished from a spherical molecule. On the other hand, the two relaxation times of the rotation of an elongated ellipsoid may differ greatly and therefore a curvature concave towards the T/η axis may be obtained.

Fig. 2 shows the plot of $(1/p - \frac{1}{3})/(1/p_0 - \frac{1}{3})$ against τ_0/ρ_0 calculated from Eqn. 28 for ellipsoids of revolution of increasing axial ratio; n_1 and n_2 have been calculated from the equations of Perrin (1934). There is a mistake in the values of n_2 in equations 96 and 96 bis in Perrin's (1934) paper. $2\rho^2 - 1$ must be changed into $1 - 2\rho^2$, the mistake coming from equation 95 where the coefficient of S in the denominator of n_2 should be $b^2a(a^2 - 2b^2)$ instead of $b^2a(2b^2 - a^2)$. From Fig. 2 it appears that the curvature is pronounced only if the accessible range of values of τ_0/ρ_0 is sufficiently large. Thus if $\tau_0/\rho_0 < 0.5$ no elongation smaller than 6 is likely to be detected, and if $\tau_0/\rho_0 < 0.2$ any elongation will fail to produce curvature.

times may be approximated by the use of Eqn. 16. In order to see the significance of f_1 and f_2 we may consider the likely case where the attached molecule executes a very rapid rotation restricted to a few degrees, independent of the slow rotation of the macromolecule. Since $3\tau_0/\rho_2 \gg 1$, the plot of $1/p$ against T/η will yield a straight line extrapolating to $1/p'_0 > 1/p_0$. After Eqn. 22 if θ is the average amplitude of the rapid rotation of the small molecule,

$$\frac{1}{p'_0} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{3 \cos^2 \theta - 1} \quad (32)$$

So that Eqn. 19 yields

$$f_2 = \frac{2}{3} \sin^2 \theta,$$

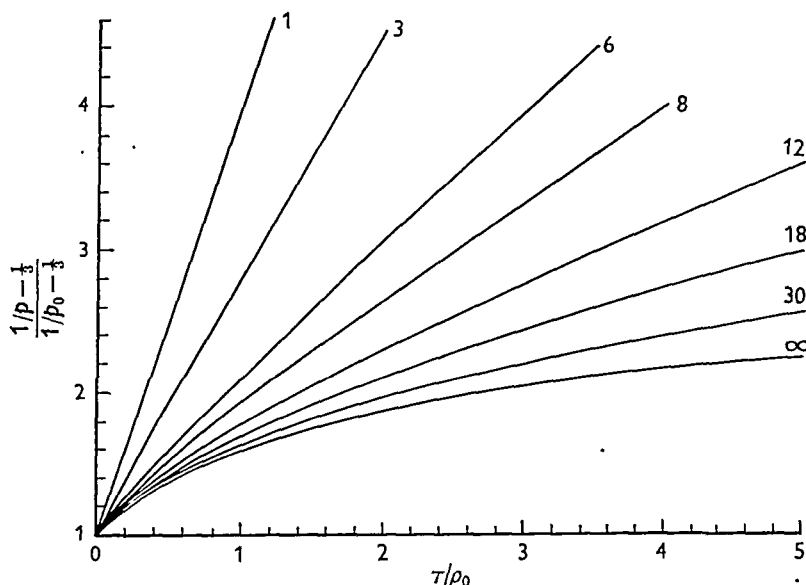


Fig. 2. Theoretical curves (Eqn. 28) for prolate ellipsoids of revolution. The number of each curve gives the elongation = $\frac{\text{long axis}}{\text{short axis}}$.

It may be concluded that in the case of molecules not very asymmetrical, i.e. the globular proteins, the simple Eqn. 1 should apply if ρ_0 is substituted by ρ_p , the harmonic mean of the principal relaxation times of the rotation.

Depolarization of the fluorescence by intramolecular rotation

The considerations that lead us to the use of Eqn. 22 allow a qualitative treatment of the case in which the small molecule attached to the macromolecule has a certain freedom of rotation with respect to the latter. If the macromolecule follows the linear law, i.e. if its depolarization can be described as resulting from one relaxation time $\rho_p = \rho_1$ and the small molecule has relaxation time ρ_2 about its conjugating bond or one nearby to it, the composition of the effects of these two relaxation

relating f_2 to the amplitude of the rapid rotation. If $(3\tau_0/\rho_2)f_1 \gg 1$ the linear law is followed, just as in the case of an impurity of low molecular weight, and the relaxation time calculated from the slope and intercept is the relaxation time of the rotation of the macromolecule as a whole.

It is evident from the preceding discussion that the study of the polarization of the fluorescence alone cannot distinguish between the effects of intramolecular rotations and the presence of a small amount of an impurity of low molecular weight. In both cases the net effect is a decrease in the value of p_0 , so that the relaxation time of the rotation of the macromolecule can be calculated without difficulty.

In the preceding discussion we have assumed that the amplitude of the internal rotations does not change with temperature. It is conceivable, however, that the amplitude allowed to such rotations

may increase due to thermal breaking of intramolecular bonds, i.e. f_2 increases with the temperature. The last two equations then give

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(\frac{1}{1 - f_2(T)} \right) \left(1 + \frac{3\tau_0}{\rho_p} \right).$$

On plotting $1/p$ against T/η a curvature convex towards the latter axis will appear, just as in the case of thermal dissociation of the molecule into rigid units. If a maximum value of f_2 is reached a straight line (s_2) or a curve concave towards the T/η axis may again obtain at higher temperature. If a definite slope is finally reached $1/p_0'$ can be determined by extrapolation. If $1/p_0 = 1/p_0'$ the curvature cannot be due to internal rotations and must result from molecular fission; s_2/s_1 , the ratio of the initial to the final slope, is then the ratio of the average relaxation times of the original and resulting particles. If $1/p_0 \neq 1/p_0'$, and if intramolecular rotations alone are the cause of the curvature, from Eqn. 21

$$\frac{s_2}{s_1} = \frac{\frac{1}{p_0} - \frac{1}{3}}{\frac{1}{p_0'} - \frac{1}{3}} \quad (33)$$

must be satisfied within the limits of the experimental error.

EXPERIMENTAL METHOD

The arrangement used for the determination of the polarization of the fluorescence is shown in Fig. 3. Light from a mercury arc is made parallel and then polarized by the

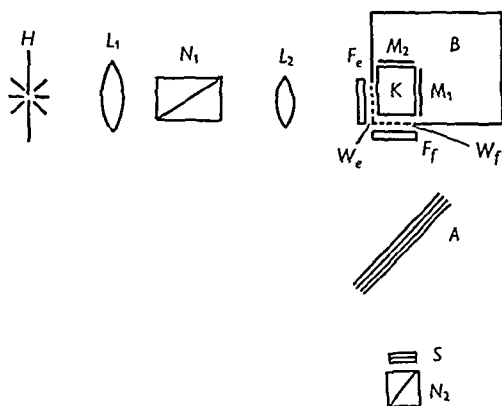


Fig. 3. Experimental arrangement for the determination of the polarization of the fluorescence. *H*, Hg discharge lamp; *L*₁ and *L*₂, lenses ($f_1 = 8$ cm.; $f_2 = 12.5$ cm.); *N*₁ and *N*₂, Nicol prisms; *K*, cell containing the fluorescent solution; *B*, water bath with glass windows; *F*_e and *F*_f, complementary filters; *A*, Arago compensator; *S*, Savart polariscope; *M*₁ and *M*₂, mirrors; *W*_e and *W*_f, windows.

Nicol prism *N*₁. A suitable filter *F*_e is placed between the latter and the cell *K* containing the fluorescent solution. This is an ordinary glass cell with parallel faces of 1 cm. depth; it

stands inside a small square water bath of some 50 ml. capacity provided with glass windows for the exciting and fluorescent light, *W*_e and *W*_f, respectively. The temperature of the bath is kept constant by means of a small heater and rheostat, or by cooling with ice as the case requires. The filter for the fluorescent light *F*_f, the Arago compensator *A* of four glass plates, the Savart polariscope *S* and its Nicol *N*₂ are mounted on a small optical bench at right angles to that carrying the lenses, the Nicol *N*₁ and the bath. The Arago compensator stands on a graduated circle and a lateral vernier allows readings of 0.1 degree. The Savart plate is mounted so that it can be tilted slightly. In this way the interference fringes can be seen moving over the field with a good improvement in sensitivity. Two small mirrors, *M*₁ and *M*₂, can be placed against the back and side of the glass cell respectively, as described by Perrin (1929). The first increases the number of excited molecules and makes the field more homogeneous while the second increases the light reaching the eye by a factor of nearly two.

Calibration of the compensator of Arago

A source of known polarization is required. This is provided by the light reflected from the face of a glass prism of known refractive index, at different incidence angles. The polarization is given by the well-known Fresnel equations (Gaviola & Pringsheim, 1924). A check on the consistency of the calibration curve can easily be done by the following method:

Let the electric vector of the linearly polarized exciting light make an angle θ with the *Oz* direction instead of being coincident with it as we have assumed till now. The fluorescent light emitted in the *Oy* direction has components *I*_θ and *I*_{θ⊥} related to *I*_∥ and *I*_⊥, the components that obtain at $\theta = 0$, in the following manner:

$$I_{\theta\parallel} = I_{\parallel} \cos^2 \theta + I_{\perp} \sin^2 \theta,$$

$$I_{\theta\perp} = I_{\perp},$$

$$\frac{1}{p_{\theta}} = \frac{I_{\parallel} \cos^2 \theta + I_{\perp} (1 + \sin^2 \theta)}{(I_{\parallel} - I_{\perp}) \cos^2 \theta} = \frac{1}{p} + \tan^2 \theta \frac{2I_{\perp}}{I_{\parallel} - I_{\perp}},$$

and remembering that $I_{\parallel}/I_{\perp} = \frac{1+p}{1-p}$, we have

$$\frac{1}{p_{\theta}} = \frac{1}{p} + \left(\frac{1}{p} - 1 \right) \tan^2 \theta. \quad (34)$$

If a solution showing considerable polarization of the fluorescence, e.g. fluorescein in glycerol, is excited with light vibrating at a variable angle θ to the *Oz* axis, and the observed values of $1/p_{\theta}$ are plotted against $\tan^2 \theta$ a straight line is obtained, the slope and intercept of which should be in the relation indicated by Eqn. 34 if no systematic error is present in the calibration of the compensator.

Some sources of error in the determination of the polarization

From Eqn. 34 it is found that if $\theta = 2^\circ$ the polarization differs by less than 1/1000 from that of $\theta = 0$. Therefore the inaccuracy in setting the plane of polarization of the exciting light should not result in any appreciable error in the determined polarization.

Convergence of the exciting beam. If a solution is illuminated with a plane polarized, parallel beam BO (Fig. 4), the direction of which has latitude θ and longitude ϕ , and the direction of the electric vector of which makes an angle θ with Oz , an observer receiving light from the source along Oy would record a polarization $p(\theta, \phi)$ defined by

$$p(\theta, \phi) = \frac{i_{\parallel} - i_{\perp}}{i_{\parallel} + i_{\perp}},$$

where i_{\parallel} and i_{\perp} are the emitted intensity components polarized along Oz and Ox respectively. The polarization p observed at right angles to the directions of propagation and vibration of the beam being as usual

$$p \equiv \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

From Fig. 4,

$$\left. \begin{aligned} i_{\parallel} &= I_{\parallel} \cos^2 \theta + I_{\perp} \sin^2 \theta, \\ i_{\perp} &= I_{\parallel} \cos^2 \phi \sin^2 \theta + I_{\perp} (\cos^2 \theta \cos^2 \phi + \sin^2 \phi). \end{aligned} \right\} \quad (35)$$

The polarization $\bar{p}(\theta, \phi)$ observed on excitation by a convergent beam of homogeneous intensity, of maximum latitude θ and maximum longitude ϕ is, by Eqn. 3,

$$\bar{p}(\theta, \phi) = \frac{\int_0^{\phi} \int_0^{\theta} (i_{\parallel} - i_{\perp}) d\theta d\phi}{\int_0^{\phi} \int_0^{\theta} (i_{\parallel} + i_{\perp}) d\theta d\phi}.$$

Introducing the values of i_{\parallel} and i_{\perp} given by Eqn. 35 into the last one and performing the integrations,

$$\bar{p}(\theta, \phi) = \frac{(I_{\parallel} - I_{\perp}) \frac{1}{4} \left(1 + 3 \frac{\sin 2\theta}{2\theta} - \frac{\sin 2\phi}{2\phi} + \frac{\sin 2\theta \sin 2\phi}{2\theta \cdot 2\phi} \right)}{(I_{\parallel} + I_{\perp}) - (I_{\parallel} - I_{\perp}) \frac{1}{4} \left(1 - \frac{\sin 2\theta}{2\theta} - \frac{\sin 2\phi}{2\phi} + \frac{\sin 2\theta \sin 2\phi}{2\theta \cdot 2\phi} \right)}. \quad (36)$$

For conical convergence $2\theta = 2\phi = x$,

$$\frac{1}{\bar{p}(x)} = \frac{1}{p} \frac{1}{\frac{1}{4} \left(1 + \frac{\sin x}{x} \right)^2} - \frac{\left(1 - \frac{\sin x}{x} \right)^2}{\left(1 + \frac{\sin x}{x} \right)^2}.$$

The second term can be neglected since it is smaller than $1/1000$ even for $x = \frac{1}{6}\pi$, and consequently,

$$\bar{p}(x) = \frac{1}{4} p \left(1 + \frac{\sin x}{x} \right)^2 \geq p \frac{\sin x}{x}.$$

Proceeding in a similar way, it is possible to show that in the case of an excitation with natural light the polarization $\bar{p}_n(x)$ is related to that observed on excitation by a perfectly parallel beam p_n , by the equation,

$$p_n(x) = p_n \left\{ \frac{\sin x}{x} - \frac{1}{4} \left(\frac{1 - \sin^2 x}{x^2} \right) \right\} \geq p_n \left\{ \frac{3 \sin x}{2x} - \frac{1}{2} \right\}. \quad (37)$$

Therefore convergences below 10° give rise to negligible errors in the observed polarization.

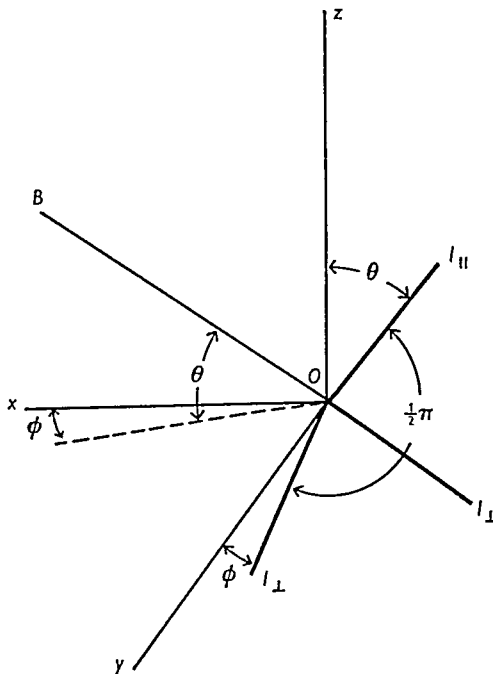


Fig. 4. Convergence of the exciting beam (for explanation see text).

Size of the source. A point in the centre of the source emits a cone of light of solid angle θ determined by the aperture of the Nicol N_2 and its

distance to the source. If the Arago compensator is rotated through an angle ϕ it is easy to see that the normal to the latter makes with the divergent light angles varying from $\phi - \frac{1}{2}\theta$ to $\phi + \frac{1}{2}\theta$. It is then possible to see interference bands due to overcompensation at one end of the source before they have been fully compensated at the opposite end. It is possible to use this fact to obtain a sharp end point in the compensation by the interposition of a lens between the source and the compensator so that the necessary divergence is obtained.

Scattering of the exciting and fluorescent light. If the filters F_e and F_f for the exciting and fluorescent light are truly complementary any scattered or reflected exciting light will not reach the eye. The scattering of the fluorescent light merits further attention. Two effects have here to be considered: the lateral scattering which would result in an increase in the observed polarization and the forward scattering which should have the opposite result. As might be

expected, the first effect is negligible compared to the second, and it is found experimentally that the polarization of the fluorescence from a turbid solution is always lower than one of the same characteristics from a transparent medium. Fortunately, the Tyndall effect shown by dilute protein solutions seems to have no detectable effect on the polarization. It is a simple matter to detect in any given case whether depolarization by forward scattering takes place. With the mirror M_2 in position, and the exciting beam traversing the middle of the cell, the average path of the fluorescent light inside the solution is about twice the path in the absence of the mirror. If the polarization of the fluorescence is the same with and without the mirror any depolarization by turbidity can be excluded.

SUMMARY

1. A simple addition law for the polarizations of several fluorescent components in solution has been derived.

2. It is shown that a system of components which differ in molecular size or in their lifetime of the excited state, but which follow independently Perrin's law of depolarization give in the plot of $1/p$ against T/η a curve concave with respect to the latter axis. (p = polarization; T = absolute temperature; η = viscosity of the solvent.)

3. An extension of Perrin's theory of depolarization to the case of ellipsoidal molecules carrying randomly oriented oscillators of absorption and emission is described. In such case the polarization of the fluorescence is an explicit function of the principal relaxation times of the rotation of the ellipsoid, the lifetime of the excited state and the limiting polarization.

4. A qualitative treatment of the depolarization by intramolecular rotations is given.

5. The experimental determination of the polarization is described and some causes of error discussed in detail.

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Polarization of the Fluorescence of Macromolecules

2. FLUORESCENT CONJUGATES OF OVALBUMIN AND BOVINE SERUM ALBUMIN

By G. WEBER*

Biochemical Laboratory, University of Cambridge

(Received 2 August 1951)

In the preceding paper (Weber, 1952) the theory of the polarization of the fluorescence given by macromolecules in solution carrying randomly oriented linear oscillators has been examined. If the relaxation time of the rotation of proteins is to be obtained by this method, the fluorescent oscillators must be rigidly bound to the molecule. Proteins as such are wholly non-fluorescent, but the radiation emitted by a stable complex of the protein with a small fluorescent molecule should have the same optical characteristics as a fluorescence of the protein molecules themselves.

* Beit Memorial Fellow.

Such a stable fluorescent complex can in theory be obtained in several ways: (i) by coupling of a small molecule through a covalent bond and subsequent elimination of the non-coupled fluorescent molecules by the ordinary methods of protein purification; (ii) by adsorption equilibrium of fluorescent molecules on the protein. It will be necessary in the latter case to determine the fraction bound to the protein by an independent method or to consider only the polarization values obtained under conditions such that the whole of the dye is bound to the protein. This last condition requires an affinity of the protein for the fluorescent molecule which is

lacking in most proteins with the conspicuous exception of serum albumin. (iii) By use of a low molecular weight substance which becomes fluorescent on adsorption to the protein. This method is free from the objections of the preceding one, and also from the objection that the protein is being chemically modified as is the case when a fluorescent molecule is attached by covalent bond. We shall show in a later paper that a family of substances exists which, although non-fluorescent in ordinary water solution, become strongly fluorescent when adsorbed.

The present paper refers to the first possibility, namely observations on stable fluorescent conjugates.

It has already been shown (Weber, 1952) that the radiation emitted by a collection of small fluorescent units attached with random orientation to macromolecules which are flat ellipsoids or prolate ellipsoids of small elongation follows a law of depolarization analogous to that first proposed by Perrin (1926) for spherical molecules, namely

$$\frac{1}{p} \mp \frac{1}{3} \approx \left(\frac{1}{p_0} \mp \frac{1}{3} \right) \left(1 + \frac{3\tau_0}{\rho_h} \right). \quad (1)$$

Here p is the degree of polarization of light emitted at right angles to the direction of the excitation, τ_0 the lifetime of the excited state of the fluorescence, and ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule. The negative signs correspond to excitation with polarized light vibrating normally to the directions of excitation and observation, the positive signs to excitation by natural light; p_0 is an empirical constant dependent often on the exciting wavelength (but not on τ_0) and in the case of macromolecules (Weber, 1952) perhaps dependent on the existence of intramolecular rotations having a relaxation time much shorter than ρ_h and largely independent of the viscosity of the solvent. This paper describes the preparation and properties of conjugates obtained by reaction of 1-dimethylaminonaphthalene-5-sulphonyl chloride with ovalbumin and bovine serum albumin. Measurements of the polarization of the radiation at different temperatures allow the validity of Eqn. 1 to be tested and the values of ρ_h in the two conjugates and in the same conjugate under different conditions to be compared.

In order to obtain reliable results the following requirements must be met:

(1) The coupling should result in a minimum of chemical change of the protein molecule.

(2) As far as possible only one type of bond should be formed between the protein and the coupled molecule. The formation of comparable amounts of different bonds may result in the protein carrying

oscillators with widely different lifetimes of the excited state.

(3) The conjugate must have a fluorescent efficiency comparable to that of the non-conjugated fluorescent substance. If the excess of the latter, which has failed to couple, has a much stronger fluorescence than the conjugate, a very exhaustive purification of the protein will be necessary in order to obtain reproducible results.

(4) A conjugating bond more stable than those responsible for the macromolecular structure of the protein may be required. Such a bond must be stable over the whole range of pH at temperatures below 100°, if the effect of temperature up to that capable of inducing denaturation is to be explored.

(5) It is desirable that the fluorescence should persist at high and low pH values. This results in a serious restriction, since in many substances the fluorescence disappears in acid or alkaline solution. If additional corrections are to be avoided in the comparisons of relaxation times obtained at different pH values, it is necessary that the quenching by acid or alkali should not affect the lifetime of the excited state of the fluorescence. No general rule can be given on this point. For example, the quenching of the fluorescence of riboflavin by acid is accompanied by decrease in the lifetime of the excited state, though not that of eosin (Weber, 1948).

The evidence to be presented in this paper shows that these conditions are met by the conjugates of serum and egg albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride. The coupling does not induce any observable denaturation of ovalbumin, as judged by the solubility at the isoelectric point; experiments with fumarase and ribonuclease indicate that the enzyme activity of conjugates containing 1-3 mol. of naphthalene per mol. of protein is comparable to that of the untreated protein. Although the formation of only one type of linkage cannot at present be demonstrated, this is rendered very likely by the large difference in affinity of the sulphonyl chloride for the —OH and =NH as compared to —NH₂ groups. The stability of the —SO₂NH— bond is well known. In no experiment was there any evidence found of the breaking down of the conjugate as shown by the appearance of the free naphthalenesulphonic acid. The fluorescence of the conjugates was not conspicuously affected by changes in the pH between 1.6 and 14.

From Eqn. 1 it is easily shown that if $p_{\min.}$ denotes the smallest polarization that can be measured with standard error ϵ the range of values of the ratio ρ_h/τ_0 that can be measured with that precision is given by

$$\frac{3+p_0}{3\epsilon} > \frac{\rho_h}{\tau_0} > \frac{3+p_0}{\frac{p_0}{p_{\min.}} - 1}.$$

In the sulphonamido conjugates studied p_0 (excitation with natural light) = 0.25; $p_{\text{mls.}}$ = 0.1; and ϵ = 0.02. Therefore,

$$55 > \frac{p_0}{\tau_0} > 2.$$

If τ_0 is of the order of 10^{-8} sec. we may expect measurable variations in the polarization for molecules having relaxation times of 10^{-7} sec. order of magnitude. In this range fall the harmonic means of the relaxation times of the rotation of most of the globular proteins studied by the dielectric dispersion method (Onley, 1942).

EXPERIMENTAL

The technique and apparatus for the polarization determinations have already been described (Weber, 1952). In the experiments described here only excitation with natural light was employed. The measurements of fluorescent intensity were made with a modified Pulfrich photometer. For both the polarizations and intensity observations the exciting light (Hg arc) was filtered through a 5850 Corning glass filter, while the fluorescence was observed through a 335 Corning glass filter. The excitation was due to the Hg lines at 366, 404 and 436 m μ . The values of p_0 obtained are therefore composite values resulting from polychromatic excitation. For reasons indicated below, the viscosities of the solutions were assumed to be those of the pure solvent. The values of Bingham and White (water) and Bingham and Jackson (sucrose solutions), as given by Bingham (1922) were used in the calculations. The absorption spectra were measured with a Beckman quartz spectrophotometer.

Materials

Ovalbumin. Preparations *A* and *C* were three and five times recrystallized ovalbumin prepared by the author (*A*) and by Dr K. Bailey (*C*). Preparation *B*, obtained from Dr A. C. Chibnall, was originally prepared by Prof. R. K. Cannan. It had been stored at room temperature as dry crystals for more than 10 years. About 10% of it was insoluble at pH 4.75, 0.1 ionic strength. Only the fraction soluble at the isoelectric point was used in the preparation of the conjugate.

Serum albumin. Crystalline bovine serum albumin (Armour Laboratories Batch nos. 10,522 and 14,656) was used throughout.

Polylysine. This was prepared by Dr C. S. Hannan (in the Press) by a modification of the method of Katchalski, Grosfeld & Frankel (1948).

Sucrose was a commercial product, the reducing power of which was equivalent to 7 parts of glucose in 100,000 (Benedict). The specific rotation was $[\alpha]_D^{20} = 66.7^\circ$ in water (24% w/v).

1-Dimethylaminonaphthalene-5-sulphonyl chloride (V). 1-Dimethylaminonaphthalene-5-sulphonic acid (I) is easily prepared by methylation of the technical 1-aminonaphthalene-5-sulphonic acid (Fussgänger, 1902). The yield is about 80% of recrystallized acid. For the preparation of the chloride 2.5 g. of the sulphonic acid are ground in a mortar with 3.5 g. of PCl₅ and the resulting yellowish melt poured on water. The insoluble chloride is exhaustively washed with water, filtered and dried over CaCl₂. The crude dry powder is

extracted successively with acetone and with m-Na₂CO₃. The alkaline extract contains usually 30–50% of unconverted acid. The acetone extract is diluted with 6 vol. of water whereupon the chloride separates as yellow or orange crystals. Yield: 25–40%, m.p. 69°. (Found: S, 12.0; Cl, 13.6. C₁₂H₁₂O₂NSCl requires S, 11.9; Cl, 13.4%.)

The sulphonyl chloride is little affected by water and can be kept for months over CaCl₂ without apparent change. It is soluble in acetone, pyridine, benzene and dioxan, insoluble in water. It reacts readily with ammonia and aliphatic amines, much less readily with aniline and very slowly with water or ethanol.

1-Dimethylaminonaphthalene-5-sulphonamide (II). 1-Dimethylaminonaphthalene-5-sulphonyl chloride (240 mg.) was dissolved in 2 ml. acetone and 1 ml. of strong ammonia was added. The sulphonamide began to crystallize immediately. Yield: 200 mg. The sulphonamide was recrystallized from ethanol in long colourless needles which showed no loss of weight after 24 hr. at 115°: m.p. 215° (decomp.). (Found: C, 57.5; H, 5.1; N, 11.2; S, 12.7. C₁₂H₁₄O₂N₂S requires C, 57.5; H, 5.5; N, 11.3; S, 12.8%.)

1-Dimethylaminonaphthalene-5-(N-phenyl)-sulphonamide (III). Freshly distilled aniline (0.1 ml.) and 269 mg. of sulphonyl chloride dissolved in 1.5 ml. of pyridine were heated in the water bath until no further change in colour was noticed (1–2 hr.). On addition of water a precipitate separated. This was dissolved by warming in 70% ethanol-water and, on cooling, the anilide crystallized in thin green needles. The crystals showed no loss of weight after 24 hr. at 115°, m.p. 141–142°. (Found: C, 66.3; H, 5.8; N, 8.7; S, 9.8. C₁₈H₁₈O₂N₂S requires C, 66.5; H, 5.6; N, 8.6; S, 9.8%.)

1-Dimethylaminonaphthalene-5-(N-benzyl)-sulphonamide (IV). Sulphonyl chloride (220 mg.) was dissolved in 2 ml. acetone and 0.5 ml. benzylamine was added. A white mass separated immediately. The mixture was taken to dryness and the residue dissolved in a little ethanol. On addition of water the sulphonamide crystallized. The pale-green needles were washed with water and recrystallized from 50% ethanol-water. Yield 190 mg., m.p. 139°. (Found: C, 66.5; H, 5.6; N, 8.3; S, 9.5. C₁₉H₂₀O₂N₂S requires C, 67.0; H, 5.9; N, 8.2; S, 9.4%.)

Preparation of the conjugates

A weight of sulphonyl chloride equal to 1–2% of the protein was dissolved in 0.5 ml. acetone and added with stirring to 10 ml. of protein solution in 0.1M-phosphate buffer, pH 7.5, or 1% NaHCO₃, kept at 0–3°. The reaction mixtures were left at 0–3° until the original turbid suspension cleared (5–12 hr.). The solutions were then centrifuged to separate suspended chloride, and dialysed with stirring in the cold against 0.2M-KCl, or 0.15M-K₂SO₄, with frequent changes of the latter until the outer liquid showed no appreciable fluorescence. (Excitation with Hg arc through Wood's filter.) This stage was reached with most proteins after about 2 days of dialysis. Serum albumin requires a considerably longer dialysis due to the adsorption of free dye. However, the adsorbed sulphonate could be easily separated by precipitation of the protein with ethanol. The conjugate was first dialysed against 0.1M-acetate buffer pH 4.7, and an equal volume of 80% ethanol was added slowly at 0°. The precipitate was collected and washed repeatedly with 50% ethanol-acetate buffer at 0° until a sample of the supernatant showed very weak, polarized fluorescence (due to a trace of the conjugate in

solution). Finally, the precipitate was centrifuged and re-dissolved in cold 0.1 M-phosphate buffer pH 7.5, and dialysed against 0.2M-KCl. One ethanol precipitation was usually enough to liberate all the adsorbed material though occasionally a second precipitation proved necessary. The combination of the sulphonyl chloride with the protein is a heterogeneous reaction, the yield of which largely depends upon the state of division of the sulphonyl chloride. If this is sufficiently fine a comparatively stable yellow suspension is obtained which, on reacting with the protein, becomes in the course of a few hours almost or completely transparent and much paler. If the initial suspension in 5% acetone water has a chloride content greater than about 0.2 mg./ml. some of it separates as crystals and the yield decreases. If the chloride content is kept below this limit and crystallization is avoided some 50–60% of it combines with the protein as judged by spectrophotometric measurements.

RESULTS

Absorption spectrum and fluorescence of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives

1-Dimethylaminonaphthalene-5-sulphonic acid and its sulphonamido derivatives show an absorption band in the 300–400 m μ . region. The maximum of this band is displaced towards the ultraviolet with increase in the ionic character of the —SO₂— group as shown in Table 1, but the molar extinction

Table 1. *Absorption spectra and limiting polarization of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives*

(p_{\max} . is the polarization observed in glycerol solution at 3°. p_0 is the limiting polarization obtained by extrapolation from measurements in glycerol at several temperatures. The concentration of the solutions in glycerol was 3×10^{-3} g./l. The absorption spectra refer to solutions in water (I), 60% ethanol (II–IV) and absolute ethanol (V).)

Sub- stance	Position of maximum (m μ .)	Absorption coefficient (cm. ² /mol.) ($\times 10^6$)	p_0	p_{\max} .
I	312	4.55	0.250	0.245
II	329	4.05	0.267	0.255
III	340	4.40	0.267	0.260
IV	332	4.46	0.268	0.255
V	369	3.73	—	—

coefficient of the maximum varies very little in the different derivatives. The integrated area under the above absorption band was found to be constant within 5% in substances II–IV. On this basis it must be expected that τ_0 is essentially the same in all these cases (Lewis & Kasha, 1945). The absolute values of the lifetime of the fluorescence obtained from the equation given by these authors were $1.1\text{--}1.2 \times 10^{-8}$ sec., although for the reasons pointed out by Lewis & Kasha (1945) no more than an indication of magnitude should be expected from this figure.

The fluorescence of the sulphonic acid and the substituted sulphonamides is quenched by acid, the region of rapid decrease of the fluorescence with pH being from 4 to 3. According to the theory of the quenching by collisions of the second kind (Wawilov, 1929) the quenching of a fluorescence with $\tau_0 \approx 10^{-8}$ requires 0.1–0.01 M concentration of quencher. As this is about 50–100 times the hydrogen-ion concentration required, it may be concluded that the quenching is non-collisional. This is confirmed by measurements of the polarization of the fluorescence of acid-quenched solutions. The substances were dissolved in 1:2-dihydroxypropane, and dry hydrogen chloride gas was passed until a convenient degree of quenching was reached. The intensities before and after quenching (I_0 and I respectively) and the corresponding polarizations (p_i and p_q respectively) were recorded. The ratio of the lifetimes of the excited state before and after quenching τ_0/τ is (Sveshnikoff, 1936)

$$\frac{\tau_0}{\tau} = \frac{1/p_i - 1/p_0}{1/p_q - 1/p_0}$$

Table 2 gives the values of I_0/I , p_i , p_q and τ_0/τ for substances I–IV. The small change in the polarization produced by the quenching reflects the long

Table 2. *Quenching of the fluorescence of 1-dimethylaminonaphthalene-5-sulphonic acid and derivatives*

(The concentration of the solutions was 6×10^{-3} g./l.)

	p_i	p_q	I_0/I	τ_0/τ
I	0.043	0.109	33	3.6
II	0.048	0.052	35	1.05
III	0.062	0.081	52	1.4
IV	0.053	0.070	60	1.4

lifetime of the non-fluorescent form, the ratio Σ/τ_0 (Weber, 1948, 1950) being 50 to 100 in the different cases quoted in the table. This long-lived non-fluorescent form may be identified with the comparatively stable $R-N^+(CH_3)_2H$, the pK of which is in the neighbourhood of 4. The values of p_0 , quoted in Table 1, were obtained by observation of the substances in glycerol at different temperatures.

The protein conjugates showed an absorption band in the 300–400 m μ . region separated from the absorption band of the protein by a well-defined minimum. Calculation of the number of molecules of sulphonamido derivative per molecule of protein has been based on the assumption that the molar absorption coefficient at the maximum of the conjugates is the same as in the sulphonamides described. An average of 4.3×10^6 cm.²/g.mol. has been used.

A. Ovalbumin conjugates

The absorption spectrum of the conjugates is shown in Fig. 1. The maximum of the band due to the presence of the dimethylaminonaphthalene groups lies at $344\text{ m}\mu$. This is noticeably displaced towards the red compared with the bands of the serum albumin (maximum at $332\text{ m}\mu$), and of the

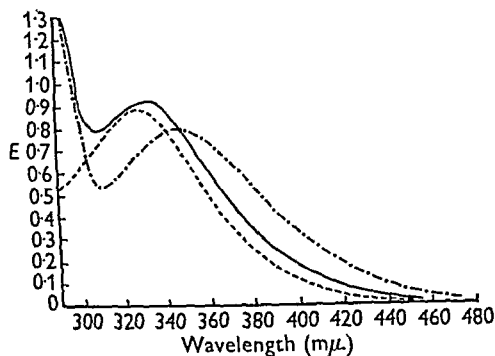


Fig. 1. Absorption spectra of conjugates. —, Bovine serum albumin (protein concentration 0.65%); ---, ovalbumin (conjugate B) (protein concentration 0.48%); ·····, polylysine (concentration 0.5%).

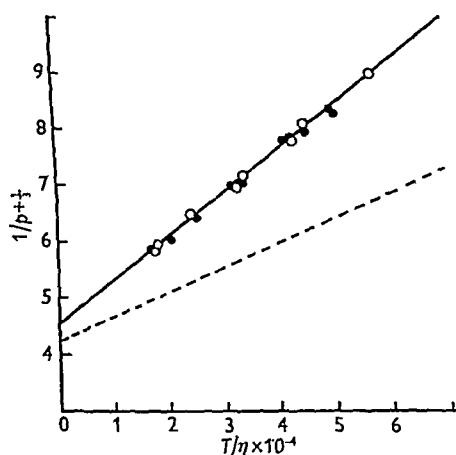


Fig. 2. Effect of temperature on the polarization of the fluorescence of ovalbumin conjugates. —○—○—, Conjugate B; —●—●—, conjugate A. The broken line is the slope observed with bovine serum albumin conjugates.

polylysine conjugate (maximum at $329\text{ m}\mu$). This causes the solutions of ovalbumin conjugates to appear yellow while the serum albumin conjugates of similar concentration are colourless. The contents of naphthalenic groups calculated for the three conjugates studied were: $A = 1.7$ mol. naphthalene/mol. protein; $B = 2.0$; $C = 2.4$. These resulted from reaction of about half the sulphonyl chloride present.

Polarization of the fluorescence

Effect of concentration. In theory, changes in the concentration of the emitting units cannot be

expected to have any effect on the polarization. This was repeatedly confirmed by experiment. Solutions of ovalbumin conjugates in 0.05M-phosphate buffer pH 7 showed no detectable change in the range of concentrations investigated, 1–0.05 % protein. Solutions of ovalbumin (0.6–1 %) dialysed against distilled water ultimately reached a pH of 4.85 and the recorded polarization was the same as with solutions in buffer, nor was this polarization changed by addition of 0.2M-potassium chloride or by dilution down to 0.1 %.

Effect of temperature. The data are shown in Table 3 and in Fig. 2. They allow the conclusion that Perrin's law of depolarization is closely followed over the range of temperatures studied, namely 3–45°. In all the conjugates studied the plotting of $1/p$ against T/η yields straight lines with regression

Table 3. Polarization of the fluorescence of ovalbumin conjugates at different temperatures

Conjugate B. Solvent: 0.04M-phosphate buffer, pH 6.85. Protein concentration: 0.24 g./100 ml.

Temp. (°)	T/η	p	
		Observed	Calculated
4.0	176	0.182	0.180
14.8	243	0.163	0.165
25.9	341	0.148	0.147
37.5	451	0.131	0.131
35.3	429	0.135	0.134
48.5	571	0.117	0.117
24.4	328	0.149	0.151
4.5	180	0.178	0.180

Solvent: 0.1 M-NaOH. Same protein concentration

3.0	170	0.181	0.182
12.1	231	0.171	0.168
26.5	346	0.146	0.147

Conjugate C. Solvent: 0.04 M-phosphate buffer, pH 6.85. Same protein concentration

3.5	171	0.182	0.181
14.9	251	0.164	0.164
8.7	206	0.175	0.174
35.0	426	0.135	0.135
25.4	337	0.150	0.148
42.4	503	0.127	0.125
42.0	498	0.127	0.126
33.9	416	0.135	0.136
5.2	184	0.181	0.179
10.0	216	0.172	0.172
18.3	179	0.163	0.168
23.9	279	0.151	0.151
31.8	395	0.139	0.139
37.8	454	0.133	0.131

The polarizations have been calculated from the equation

$$\frac{1}{p} = 4.22 + 0.75 \times 10^{-4} \frac{T}{\eta}$$

The measurements are given in the order in which they were performed.

Conjugate	$1/p_0$	$b \times 10^6$	$\beta \times 10^6$
A	4.19 ± 0.06	76.7 ± 1.8	18.2 ± 0.5
B	4.20 ± 0.06	75.6 ± 1.8	17.9 ± 0.5
C	4.24 ± 0.05	75 ± 1.3	17.7 ± 0.4

coefficients with a standard error of about 2%. According to Eqn. 1 the regression coefficient b contains the factor $1/p \mp \frac{1}{3}$, so that for two conjugates having intercepts $1/p_{01}$ and $1/p_{02}$

$$\frac{p_2}{p_1} = \frac{b_1 | 1/p_{01} \pm \frac{1}{3} |}{b_2 | 1/p_{02} \pm \frac{1}{3} |}.$$

Therefore, if the linear law of depolarization is followed, the characteristic quantity for the conjugate is

$$\beta = \frac{b}{1/p_0 \pm \frac{1}{3}}.$$

This is given in Table 3 for the egg albumin conjugates together with the propagated error

$$\frac{\Delta\beta}{\beta} = \sqrt{\left\{ \left(\frac{\Delta b}{b} \right)^2 + \left(\frac{\Delta(1/p_0 \pm \frac{1}{3})}{1/p_0 \pm \frac{1}{3}} \right)^2 \right\}}.$$

The thermal effects were perfectly reversible. No detectable changes in the polarization were found after keeping the protein for 1 hr. at 45°.

The use of distilled water or dilute salt solutions from 2 to 50° allows a range of values of T/η between 1.6 and 6×10^4 . To reach lower values of T/η , it is necessary to increase the viscosity of the solution by addition of a foreign substance. It is very doubtful whether the microscopic viscosity, which alone determines the resistance to the molecular rotations, can be equated in all cases with the viscosity measured by flow. To test this point the effect of electrolytes and of sucrose on the polarization was tried. Using sodium chloride and potassium chloride it was found that the increase in polarization produced by addition of electrolyte was much smaller than predicted by Eqn. 1. Concentrations below 1M yielded polarization values which were indistinguishable from those in distilled water. Therefore in the calculation of T/η of solutions in dilute buffer ($M \leq 0.2$) the viscosity of the pure solvent was used in every case. Better agreement with Eqn. 1 was observed when sucrose was used to increase the viscosity of the solvent particularly in the case of neutral solutions of serum albumin as described later in this paper. The viscosity of a 60% (w/v) sucrose solution at 3–5° is of the order of poises (Bingham, 1922). From Eqn. 1, if $3\tau_0/\rho_h$ in water is 1 or less, its value in 60% sucrose should be negligible compared to 1 and the polarization observed under this condition should not differ from p_0 , as determined by extrapolation, if the linear law is followed throughout the range of T/η . Native ovalbumin conjugates dissolved in 60% sucrose (w/v) yielded $p = 0.236 \pm 0.004$, which is within the errors of the experiments the same as the extrapolated p_0 . Therefore there is no detectable curvature and according to Eqn. I, 29,* the relaxa-

tion time calculated from the slope and intercept is ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoid. Consequently

$$\frac{\tau_0}{\rho_h} = \frac{1}{3} \frac{1/p - 1/p_0}{1/p_0 + \frac{1}{3}} = \frac{bT/3\eta}{1/p_0 + \frac{1}{3}} = \frac{\beta T}{3\eta}$$

from Eqn. 1. If $\beta = 1.68 \times 10^{-5}$ and T/η at $25^\circ = 3.33 \times 10^4$,

then $(\tau_0/\rho_h)_{25^\circ} = 0.186$. According to Oncley (1942), the harmonic mean of the principal relaxation times of the rotation of ovalbumin at 25° in water is 7.55×10^{-8} sec. so that $\tau_0 = 1.4 \times 10^{-8}$ sec. It will be convenient to assume in the future a value of 1.4×10^{-8} sec. for the lifetime of the excited state of the fluorescence of the conjugates. By introducing it in Eqn. 1 the relaxation times of the rotation of the conjugate molecules can be calculated, provided the depolarization follows the linear law.

Effect of pH on the relaxation time of the rotation

Changes of the pH of the solution between 1.5 and 13 produce no immediate changes in the polarization of the fluorescence. This is shown in Fig. 3

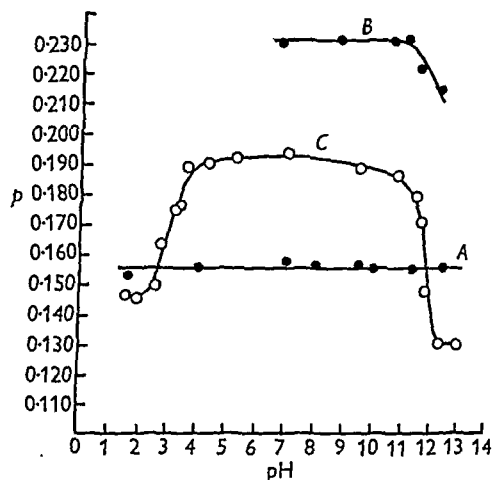


Fig. 3. Effect of pH on the polarization of the fluorescence. A, native ovalbumin; B, heat denatured ovalbumin; C, bovine serum albumin.

which refers to measurements done at 19°. The protein was dialysed against 0.02M-potassium chloride and the pH adjusted, by the addition of hydrochloric acid or sodium hydroxide, to the required value which was measured by glass electrode. The polarization at each value was recorded as soon as possible afterwards, and the measurement repeated at intervals during at least 1 hr. During this time no change whatsoever was found in solutions between pH 3 and 13. At pH values between 1 and 3 a slow increase in the polarization was noticed. This increase was much faster if the solutions were kept at 37°. When the solutions kept for some time at pH 2 were adjusted to the isoelectric point a precipitate of

* This refers to Eqn. 29 of the first paper of this series (Weber, 1952). This notation is used throughout.

denatured protein appeared. The supernatant solution showed the polarization value corresponding to the native protein. This acid denaturation was studied more fully and the results are described below.

Time course of the acid denaturation

Fig. 4 shows the changes in the polarization during the course of the denaturation of 0.6% solutions of ovalbumin kept at pH 1.53–1.65. The protein was dialysed against distilled water, and then diluted to the required concentration. The

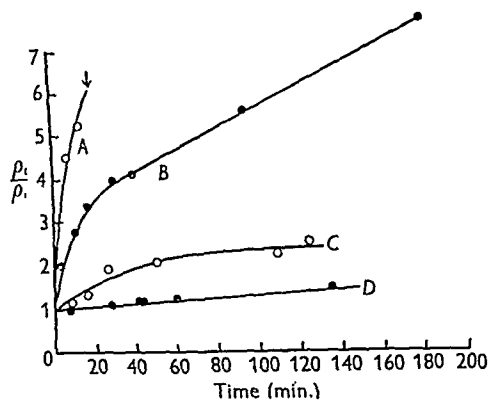


Fig. 4. Time course of the acid denaturation of ovalbumin. A, 0.2M-KCl, 30°; B, salt-free, 30°; C, 0.2M-KCl, 3°; D, salt-free, 3°.

polarization of the fluorescence was then determined at two temperatures, and finally the pH was adjusted to the required value by addition of M-hydrochloric acid. The cell was maintained at constant temperature ($\pm 0.5^\circ$) and readings were made at intervals during 2 hr. The ratio of the apparent relaxation time p_t to the initial relaxation time p_i is given by the simple equation

$$\frac{p_t}{p_i} = \frac{1/p_t - 1/p_0}{1/p_i - 1/p_0},$$

where p_i is the initial polarization and p_t its value at time t . It is assumed that the lifetime of the excited state does not change with pH. This is substantiated by the fact that in the protein solution kept at 3° in the absence of salt the value of p during the first 10 min. was the same as before the addition of the acid. In the other cases the course of the reaction shows that the value of p at zero time cannot differ greatly from that at neutral pH. Moreover, as shown by Table 2, the quenching of the fluorescence of several derivatives of 1-dimethylaminonaphthalene-5-sulphonamide by H ions does not result in any rapid change of the lifetime of the excited state of the fluorescence.

The curves of Fig. 4 show that the increase in the apparent relaxation time of the particles is much faster at the higher temperature, and faster in the

presence than in the absence of salt. This applies also to the final values reached after several hours. At 30° , in the presence of salt (0.2M-potassium chloride), a copious precipitate occurred after a few minutes (arrow in Fig. 4) and no further measurements were possible.

Alkaline denaturation

The values quoted in Table 3 show that ovalbumin dissolved in 0.1M-sodium hydroxide had the same relaxation time of the rotation as at neutral pH. On adjusting the pH to the isoelectric point, the bulk of the protein separated. Therefore in alkaline solution, although the protein was denatured just as readily as in acid, no increase in the relaxation time was observed. When the isoelectric precipitate was redissolved in 0.1M-phosphate buffer, pH 7.8, considerable increase in the relaxation time of the rotation was observed. Even in 0.1M-sodium hydroxide the average relaxation time of the dissolved isoelectric precipitate was considerably higher than the native protein.

Solutions of ovalbumin kept at pH 12–13 for 24 hr. showed a decrease in the relaxation time, probably due to partial hydrolysis of the protein. Such decrease was never observed in experiments of short duration.

Urea denaturation of ovalbumin

To a solution of ovalbumin in distilled water (pH 4.9) was added 0.49 g. urea/ml. of original solution. After standing for 4 days at 0° the urea was dialysed away and the solution finally equilibrated against 0.05M-phosphate buffer, pH 7.3. The crystal clear solution had a concentration of 0.12% protein. Fig. 5 shows that the average relaxation time of the rotation of the particles had increased by about eight times as compared with that of the native protein. On heating the solution the polarization decreased as expected from the linear law.

Heat denaturation

Dilute solutions of ovalbumin (0.1–0.2%) in 0.05M-phosphate buffer, pH 7, boiled for a short time showed strong increase in the polarization of the fluorescence (Fig. 5). After boiling the solutions for 5 min. the absorption spectrum showed a maximum at $332 m\mu$. and was now practically coincident with the spectra of the serum albumin conjugates. If the solution was boiled for 1 min. a hybrid curve showing two maxima at 332 and $342 m\mu$. was obtained (Fig. 6).

The difference of absorption curves of native egg and serum albumins is not likely to be due to any difference of the covalent attachment of the sulphonamido groups. Since denatured ovalbumin, in common with other denatured proteins (Oster & Grimsson, 1949), resembles serum albumin (as

native egg albumin does not) in having a great capacity for adsorption of dyes, the identity of the absorption curve of their conjugates may be accounted for by secondary binding of the sulphon-amido groups, which would be absent in conjugates

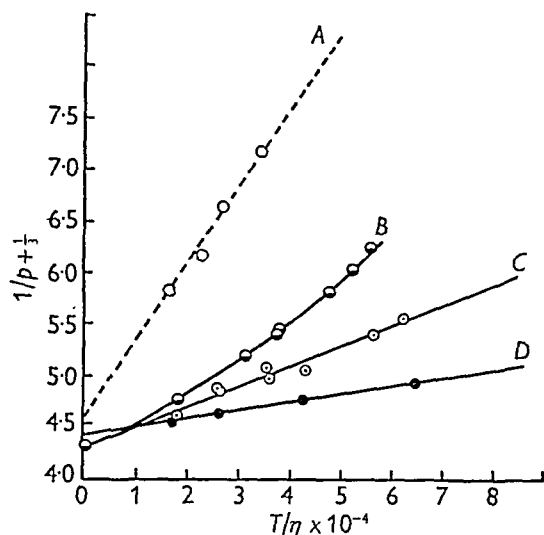


Fig. 5. Polarization of conjugates of denatured ovalbumin. A, ovalbumin denatured in alkali (dissolved in 0.1M-NaOH); B, heat-denatured in 0.04M-phosphate buffer, pH 7.2; C, denatured by urea (solvent: 0.04M-phosphate buffer, pH 7.2); D, denatured by acid, salt-free, pH 1.7. The broken line is the slope corresponding to the native ovalbumin.

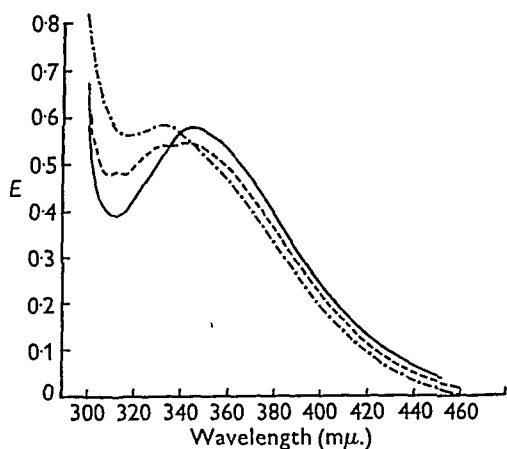


Fig. 6. Change of absorption spectrum of ovalbumin conjugates on heat denaturation. —, native ovalbumin; ----, boiled for 1 min.; - · - · -, boiled for 5 min. Protein concentration 0.36%.

of native ovalbumin. This is supported by the lower value of p_0 for native ovalbumin (0.236) than that for serum albumin (0.257), and the increase of the former value to 0.254 ± 0.04 on heat denaturation (measured in 60% sucrose).

B. Bovine serum albumin conjugates

Absorption spectrum. The conjugates studied contained 1–3 mol. of naphthalene per mol. of protein of molecular weight 70 000. The absorption spectrum is shown in Fig. 1. The maximum of absorption is at $332 \text{ m}\mu$, very nearly that obtained with a polylysine conjugate, though shifted slightly to longer wavelength. Such change has been found in the absorption spectra of many dyes adsorbed on serum albumin (Laurence, 1952). This is consistent with the secondary binding of covalently attached molecules suggested above. In the conjugate, the absorption spectrum of which is shown, there were 2.3 mol. of naphthalene per mol. of protein, resulting from the reaction of 60% of the added chloride.

Effect of concentration on the polarization of the fluorescence. Solutions of 1–0.02% showed no appreciable difference as regards the polarization of the fluorescence, both in distilled water (pH 5.25) and in 0.05M-phosphate buffer, pH 7.

Temperature effect on the polarization. Solutions of conjugate at pH 6–8 followed the linear law between 3 and 50° . Above 50° a marked departure from linearity took place. The temperature effects were perfectly reversible. Keeping the protein solutions at this pH for 1 hr. at 59° did not result in any significant change in the observed polarization. Several conjugates prepared over a period of more than a year yielded entirely reproducible results. The regression coefficients of $1/p$ upon T/η varied from 4.18×10^{-5} to 4.36×10^{-5} in the different cases with an average of 4.25 ± 0.1 . The value of $1/p_0$ was 3.90 ± 0.06 . The data are given in Table 4 and in Fig. 7.

Polarization of the fluorescence in sucrose solutions. The data are shown in Table 4. It appears that the values of the polarization in 20% (w/v) sucrose is what would be expected from Eqn. 1 if the viscosity measured by flow is introduced. The polarization calculated using $b = 4.25 \times 10^{-5}$, obtained from measurements in water, agree with the observed values within the experimental error.

Fig. 8 gives the plot of $\frac{1/p + \frac{1}{2}}{1/p_0 + \frac{1}{2}}$ against τ_0/ρ_0 ; where ρ_0 is the relaxation time of a sphere of volume equal to that of the serum albumin molecule. If this has anhydrous molecular weight M and partial specific volume δ , and the hydration is H g. of water per g. of anhydrous protein

$$\rho_0 = \frac{3\eta}{RT} M(H + \delta).$$

Introducing $\tau_0 = 1.4 \times 10^{-8}$ sec. from the measurements with ovalbumin $M = 7 \times 10^4$; $\delta = 0.75$; and $H = 0.15$ from the recent measurements of Haggis, Buchanan & Hasted (1951), we have

$$\frac{\tau_0}{\rho_0} = 6.15 \times 10^{-6} \times \frac{T}{\eta}.$$

Table 4. Polarization of the fluorescence of bovine serum albumin conjugate at different temperatures

(Conjugate containing 2.3 mol. naphthalene/mol. protein. Solvent: 0.05M-phosphate buffer, pH 6.77. Protein concentration: 0.12%.)

Temp. (°)	$T/\eta \times 10^{-2}$	p	
		Observed	Calculated
3.4	173	0.215	0.215
10.7	221	0.210	0.206
23.5	320	0.188	0.189
30.3	380	0.182	0.180
35.4	431	0.176	0.174
44.5	526	0.163	0.162
38.9	465	0.173	0.169
51.5	606	0.155	0.153
55.7	657	0.148	0.148
58.4	690	0.140	0.146
14.5	248	0.200	0.201
3.0	170	0.215	0.216
20.2	293	0.192	0.193
50.2	592	0.152	0.155
54.8	646	0.145	0.149
58.8	696	0.138	0.144

Same conjugate. Sucrose added to 20% (w/v).

3.5	83	0.237	0.236
16.7	135	0.225	0.224
27.6	188	0.214	0.213
35.1	235	0.206	0.204
39.5	260	0.200	0.198
48.1	319	0.190	0.190
53.5	365	0.181	0.182

The polarizations have been calculated from the equation

$$\frac{1}{p} = 3.90 + 0.433 \times 10^{-4} \frac{T}{\eta}.$$

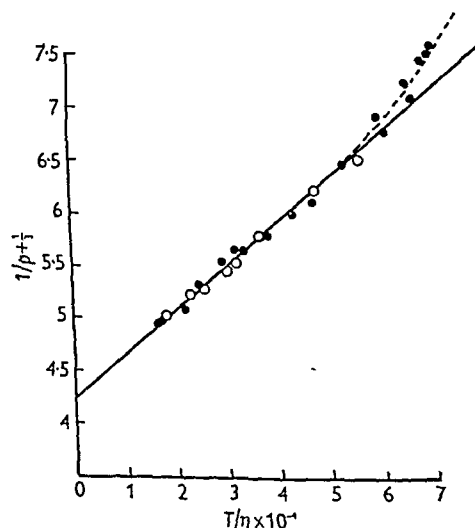


Fig. 7. The effect of temperature on the polarization of the fluorescence of bovine serum albumin conjugates. The open and filled circles correspond to two different preparations. The solid line is the regression line obtained from the observations at temperatures below 50°.

The figure shows that an axial ratio of 4 would account well for the observed values. If $H=0.3$ the axial ratio would be about 3 and 5 if $H=0$.

The polarization recorded in 60% sucrose at 2° was 0.257 ± 0.002 . Within the limits of the experimental error this is the same as the value obtained by extrapolation (0.256).

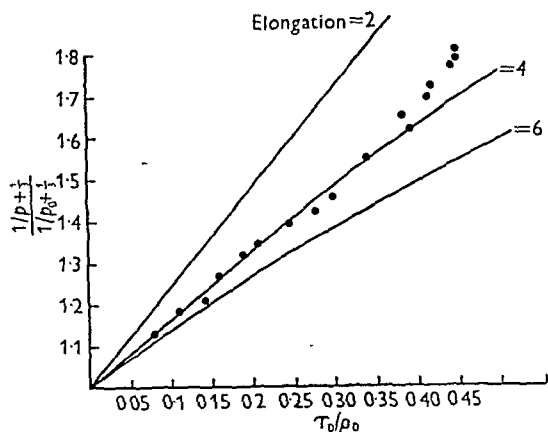


Fig. 8. Abscissa: τ_0/p_0 . Ordinate: $\frac{1/p + 1/2}{1/p_0 + 1/2}$, as explained in the text. The curves are the theoretical for ellipsoids of elongation 2, 4 and 6 (Weber, 1952).

The harmonic mean of the relaxation times of the rotation at 25° in water can be determined from Eqn. 1 in which $\tau_0 = 1.4 \times 10^{-8}$ sec. We thus obtain $\rho_h = 1.27 \times 10^{-7}$ sec., while Oncley (1942) gives $\rho_h = 1.24 \times 10^{-7}$ sec. from measurements of the dielectric dispersion with horse serum albumin.

Observations on bovine serum albumin conjugate in acid solution

If a 0.1% solution of conjugate is thoroughly dialysed against distilled water and the pH adjusted to 1.8–2 by addition of a small amount of M-hydrochloric acid the polarization of the fluorescence drops from 0.196 (at 18°) to 0.147. Measurements at different temperatures at this pH yielded

$$\begin{aligned} b &= (9.68 \pm 0.2) \times 10^{-5}; \\ 1/p_0 &= 4.08 \pm 0.07; \\ \beta &= (2.2 \pm 0.06) \times 10^{-5}. \end{aligned}$$

In 60% sucrose $p=0.245$ in good agreement with the extrapolated p_0 . The results are shown in Fig. 9. After neutralization by dialysis against 0.05M-phosphate buffer, pH 7, the observed polarizations yielded $b=4.3 \times 10^{-5}$; $1/p_0=3.95$; $\beta_0=1.02 \times 10^{-5}$, showing that the changes in polarization with pH were perfectly reversible.

The intensity of the fluorescence of the salt-free conjugates at pH 2 was 60% of the intensity at pH 7, while the absorption spectrum showed no conspicuous change. The observations on the ovalbumin

conjugates, together with those on the quenching of the fluorescence of the sulphonamido derivatives, show that no change in the lifetime of the excited state is to be expected. It must be concluded that the changes in polarization are due to a change in the relaxation time of the rotation. In turn, this cannot be due to a simple change in shape of the serum albumin neutral molecule, if this is an ellipsoid of axial ratio 5 or less. On changing into a sphere, such

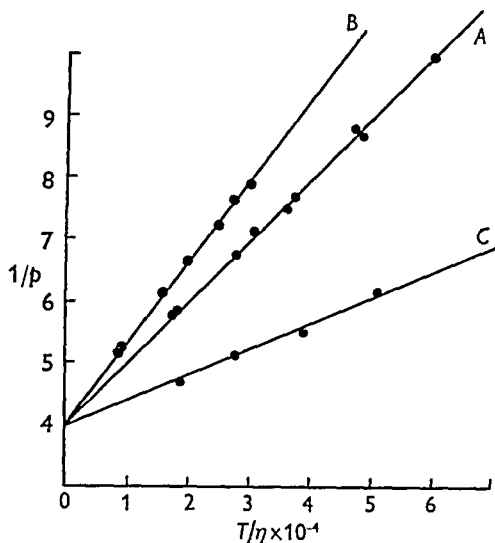


Fig. 9. *A*, polarizations of bovine serum albumin (0.12%) in salt-free pH 1.9 solution; *B*, 20% (w/v) sucrose at pH 1.9; *C*, solution *A* neutralized to pH 7.

an ellipsoid will have a relaxation time of 0.52 times the original value, while the observed ratio of β_0/β is 0.46. In order that this may be explained by a change in shape the neutral molecule would have to be an ellipsoid of elongation 8 or more or a flat molecule of axial ratio greater than 5. The absence of curvature in the plot of $1/p$ against T/η (Fig. 8) excludes the former, while the data of the dielectric dispersion (Oncley, 1942) do not support the latter. We may then conclude that molecular fission is the cause of the observed changes in the polarization. The number of resulting particles cannot be easily decided because of the unknown shape of the sub-units. If these are less asymmetric than the parent molecule their number cannot be other than two, but if their asymmetry is greater a larger number is possible. The fact that the resulting particles may not all be of equal size should, within wide limits, have little influence on the observed slope, provided that the original and resulting particles have similar shape. If we assume that the groups that can react with the sulphonyl chloride have equal affinity for this reagent, the particles will contribute to the total fluorescent intensity in proportion to the number of such groups that they possess. In the absence of more detailed information it is convenient

to assume that, in a solution of particles that have reacted together with the sulphonyl chloride, f_i the contribution of each species to the total emitted intensity is proportional to its relaxation time. Therefore

$$f_i = \frac{N_i \rho_{hi}}{\sum_i N_i \rho_{hi}},$$

where N_i is the number of molecules of relaxation time ρ_{hi} . The weighted harmonic mean of the relaxation times is

$$\bar{\rho}_h = \frac{1}{\sum_i \frac{f_i}{\rho_{hi}}} = \frac{\sum_i N_i \rho_{hi}}{\sum_i N_i} = \bar{\rho}_a,$$

where $\bar{\rho}_a$ is the number average of the relaxation times. Thus, if the original and resulting particles have the same shape, the ratio of the β quantities is the number of units into which the original molecule has dissociated.

The polarizations obtained at pH 2 in 20% (w/v) sucrose (Fig. 9) were systematically lower than in water at equal values of T/η . The sucrose points fall evenly on a straight line with $b = 1.30$; $1/p_0 = 4$; $\beta = 2.70$. The difference from the values in water may be due to more complete dissociation in sucrose (hydrogen bonds?) or to differences between the microscopic viscosity and the viscosity measured by flow. It may be pointed out that at pH 2 a small percentage of the sucrose present was hydrolysed and therefore a chemical reaction of the hexoses with the protein cannot be excluded.

Effect of salt on the acid dissociation. In the presence of M-potassium chloride, or 0.1 M-potassium sulphate the dissociation did not take place when the pH was brought from neutrality to 1.9. If the salt was added after the dissociation was obtained, complete reversal was observed, the polarization increasing to the original value in neutral solution.

Observations on bovine serum albumin in alkaline solution

0.5 ml. of 0.5% salt free conjugate was mixed with 2 ml. of 0.1 M-sodium hydroxide and the polarizations at different temperatures recorded. When measurements at temperatures higher than 25–30° were attempted an irreversible fall in the polarization was noticed. This was also noticed at lower temperatures if the alkali was left to act for a longer time (i.e. 12 hr. at room temperature). The polarization measurements below 28° were temperature reversible and could be repeated with several conjugates within 5%. As shown in Fig. 10, these measurements yielded $b = 1.19$; $1/p_0 = 4.34$; $\beta = 2.56$. The polarization in 60% sucrose was 0.230 in excellent agreement with the extrapolated value.

Solutions left for an hour at 18°, and then neutralized, showed that the dissociation in alkali is

completely reversible as regards the relaxation time of the rotation. Solutions left for 10 hr. in alkali at 4° showed when neutralized a fall in the polarization indicating that if the action of the alkali is prolonged the reaction is no longer wholly reversible.

The slope and intercept of the solutions in alkali show differences from the values in acid. The comparatively minor differences in the values of β (0.46 and 0.40 respectively) can be due to a variety of causes, the analysis of which is not possible with the sole resources of this method. As regards the decrease in p_0 observed in alkali, several sources (Klotz, 1949; Laurence, 1952) indicate that the binding power of serum albumin is much diminished in alkaline solution. It may then be expected that

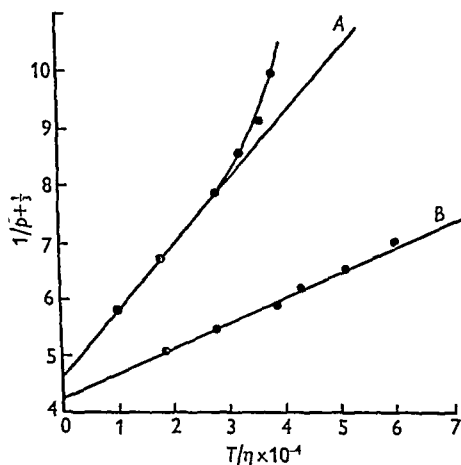


Fig. 10. *A*, polarizations of bovine serum albumin in 0.1 M-NaOH; *B*, a similar solution, kept for an hour at room temperature and then neutralized.

the freedom of rotation of the attached molecule should be larger in the latter case, leading to a decrease in the limiting polarization (Weber, 1952). The alkaline dissociation is largely unaffected by salt.

Effect of pH on the relaxation time of the rotation

Fig. 3 shows the effect of pH on the polarization of the fluorescence of 0.1% salt-free conjugates. The pH changes were obtained by addition of dilute hydrochloric acid or sodium hydroxide respectively and measured by glass electrode. The following points were noticed: (i) The shift in the polarization of the fluorescence with pH was complete in a matter of minutes, this being the time necessary to take a series of readings. In no case could a time effect be detected, even in solutions kept at 3°. (ii) Threefold dilution at any pH value did not result in any conspicuous change in the polarization. (iii) The main changes took place between pH 4 and 2.5 on the acid side, between

9.5 and 11.8 on the alkaline side. No further change was observed below pH 2 or above pH 12. (iv) The neutralized solutions showed no detectable difference from the original neutral solution. The most important fact to be noticed here is the complete correspondence of the regions of rapid change of the polarization with the main regions of titration of the protein.

Effect of urea treatment of the serum albumin conjugates

After treatment with 6M-urea and subsequent removal of the latter by dialysis, the plot of $1/p$ against T/η , as shown in Fig. 11, gave a slope of

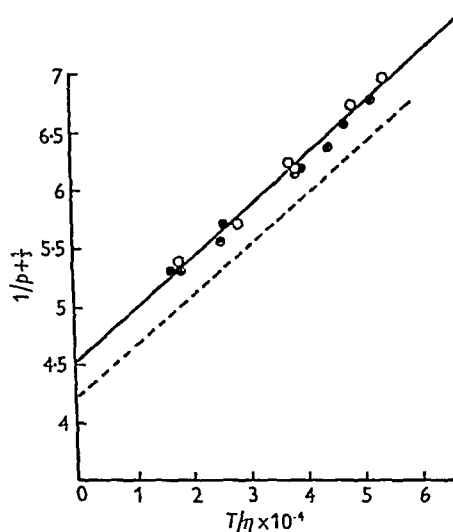


Fig. 11. Bovine serum albumin regenerated from 6M-urea. The filled and open circles correspond to two independent experiments. The broken line is the slope of the native albumin.

0.40–0.42 in the different preparations, and intercept varying from 4.28 to 4.10. The slope was therefore not significantly different from that of the native protein, while the intercept was slightly, though consistently, higher. In 60% sucrose the observed polarization was 0.245 ± 0.005 . The protein regenerated from urea has been found by sedimentation studies (Putnam, Erickson, Volkin & Neurath, 1943) to have a molecular weight equal to the native, and the observations here reported agree with it. The increase in the intercept can be interpreted as an increase in the rotational freedom of the coupled molecule due to decrease in the binding power of the protein, and a study of this property in urea-regenerated serum albumin may decide on the validity of this interpretation.

DISCUSSION

As described in the experiments, the sulphonyl chloride reacted very readily with the primary amino groups so that the formation of sulphon-

amido linkages as the most important product of the reaction with the protein offers little doubt. The absorption spectrum of the sulphonyl chloride presents a maximum at $369\text{ m}\mu$. and therefore, if any important amount of unreacted chloride were to remain adsorbed to the protein, a second maximum or an inflexion should appear in that region of the absorption spectrum of the conjugates. Such inflexion was observed in mixtures of proteins and chloride at the beginning of the reaction, but as this proceeded the inflexion decreased and disappeared.

Perrin's law of depolarization is followed by the serum albumin and ovalbumin conjugates within the limits of the experimental error. If the validity of Eqn. I, 28 is accepted, the absence of visible curvature may be taken as an indication that the molecules are flat or only moderately elongated. In all cases where the straight line law was followed excellent agreement was found between the limiting polarization p_0 as determined by extrapolation and the polarization observed in 60% sucrose solution. The differences in the value of p_0 in the different conjugates may be attributed to the varying degree of rotation about its conjugating bond allowed to the coupled molecule. The calculation of absolute values of the mean relaxation time of the rotation requires τ_0 to be known. Relative values of ρ_h may, however, be calculated if τ_0 is assumed to be the same in the different conjugates. This can only be decided by direct measurement of τ_0 , but the data presented show that the calculation of this quantity using the available figures for the relaxation times of the rotation of ovalbumin and serum albumin leads in both cases to the same value, namely $\tau_0 = 1.4 \times 10^{-8}$ sec.

It is also assumed that no change takes place in the lifetime of the excited state between pH 1.5 and 14. Ovalbumin conjugates give the same polarization between these extreme values provided the observations in acid are done rapidly and at low temperature to avoid denaturation. The conjugates at pH 1-2 show a decrease in the fluorescence intensity and the lack of change in the lifetime of the excited state must be attributed to the long lifetime of the non-fluorescent form (Weber, 1948). A study of the quenching of the fluorescence of the sulphonamido derivatives of 1-dimethylaminonaphthalene-5-sulphonic acid makes clear that such is the case and that moderate degrees of quenching should not alter sensibly the value of τ_0 . It is worthy of note that the quenching of the substituted sulphonamides took place at pH 3-4, while the conjugates showed little quenching (about 30%) at pH 1.5. This difference is readily explained by the fact that at pH lower than the isoelectric point the ionic atmosphere of the protein will contain fewer hydrogen ions than the bulk of the solvent. The

fluorescent intensity of the conjugates of serum albumin was considerably greater than those of native ovalbumin. Here once more the notion of a tautomeric non-fluorescent modification endowed with long life is probably valid.

Ovalbumin conjugates. The most important fact to emerge from the study of these conjugates is that reversible dissociation of the protein, by acid, alkali, or heat, was never seen and that the relaxation time increased considerably on denaturation by acid, alkali, heat, or urea. This increase in the observed relaxation time cannot be explained by an increase in the axial ratio of the particles alone. If the original quasi-spherical ovalbumin molecule is drawn into an ellipsoid of infinite axial ratio, Eqn. I, 28 shows that the apparent relaxation time cannot increase by a factor greater than 3. In the experiments, however, as shown in Figs. 4 and 5, increases of eight times and more were observed. These results lend themselves to a simple and complete interpretation if aggregation of denatured molecules is assumed to be the main cause of the increase in the relaxation time of the rotation. In the experiments on the time course of the acid denaturation, a visible precipitate appeared only in the case where the rate of increase of the relaxation time was a maximum, suggesting a continuous transition between the postulated aggregates and the precipitate. The isoelectric precipitation of denatured ovalbumin may be considered as the continuation of the aggregation on to a macroscopic scale.

If the aggregation is due to interactions which, like hydrogen bonds and van der Waals forces, decay rapidly with the distance, the main factor determining the growth of the particles will be the extent to which the electrostatic repulsion between the molecules can offset the thermal agitation and thus prevent the approach and interaction that leads to permanent binding (Verwey & Overbeek, 1948). The addition of electrolyte by decreasing the thickness of the double layer lets the particles grow larger and in the experiment at 30° the co-operative effect of salt and temperature allowed indefinite aggregation and flocculation to take place. The acid denaturation was accompanied by immediate increase in the relaxation time, while the protein denatured by alkali did not show it until the pH was brought near the isoelectric point. Moreover, when dissolved in acid, the alkaline denatured ovalbumin showed also increased relaxation time. A likely explanation of these facts is that the aggregation in acid is due to hydrogen bonds mainly from the uncharged carboxyl groups. No comparable source of hydrogen bonds exists in the alkaline protein.

The aggregation of ovalbumin in acid has been shown by Huang & Wu (1930) by osmotic pressure measurements.

Serum albumin conjugates. In contradistinction to ovalbumin the relaxation time of the rotation showed marked dependence on the pH of the solutions. The data presented show that the changes cannot be attributed to increase in τ_0 or to a simple change in shape of the serum albumin molecules, and that reversible dissociation into sub-units must be admitted. The study of this dissociation will be the object of another paper. It need only be indicated here that the departure from the linear law above 50° observed in neutral solution (Figs. 7 and 8) can be explained by thermal dissociation of the molecule.

SUMMARY

1. The preparation of stable fluorescent conjugates of ovalbumin and bovine serum albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride is described.

2. It is shown that the fluorescence of solutions of the purified conjugates follow Perrin's law of depolarization, yielding straight lines when the reciprocal of the polarization is plotted against the ratio of the absolute temperature to the viscosity of the solvent.

3. The slopes of the conjugates studied are reproducible within 2%, and for ovalbumin and bovine serum albumin at neutral pH are in the ratio of the harmonic mean of the principal relaxation times of the rotation as required by theory.

4. The polarizations observed in 60% sucrose

solution are, in all cases studied, in excellent agreement with the limiting polarization obtained by extrapolation.

5. The relaxation time of the rotation of ovalbumin is shown to be independent of pH within a wide range (pH 1.5-14).

6. The denaturation of ovalbumin by acid, urea or heat results invariably in an increase in the relaxation time of the particles. The effect of temperature and of neutral salts on the acid denaturation has been studied.

7. The polarization of the fluorescence of bovine serum albumin conjugates is independent of pH between 4 and 9, but falls rapidly outside this region to reach lower stable values at pH 2 and 12 respectively. It is shown that only reversible dissociation into sub-units can explain these facts.

8. Bovine serum albumin regenerated from urea does not differ appreciably from the native species as regards particle size.

9. The preparation and characterization of 1-dimethylaminonaphthalene-5-sulphonyl chloride and of several sulphonamido derivatives are described.

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A Study of the Adsorption of Dyes on Bovine Serum Albumin by the Method of Polarization of Fluorescence

By D. J. R. LAURENCE*

Biochemical Laboratory, University of Cambridge

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The ability of serum to bind small molecules was brought into prominence by the work of de Haan (1922) on the excretion of vital dyestuffs by the kidney. Grollman (1925) later showed by ultra-filtration that serum albumin possesses the property not possessed by ovalbumin, serum globulin, or gelatin, of adsorbing phenol red at alkaline pH. He showed that all the proteins studied bound the dye when the dye and the protein were of opposite electrical charge, but that serum albumin could also bind the negatively charged dye when it was itself negatively charged, adsorption decreasing rapidly, however, above pH 8. Bennhold (1932) showed that a variety of dyes and substances of pharmacological value was carried by the albumin fraction of serum and proposed for the protein a transporting function in the animal body. Recent studies on the binding by the serum albumin fraction (Smith & Smith, 1938; Rawson, 1943; Chow & McKee, 1945) and especially the detailed studies of Murray Luck and his co-workers on the stabilization of serum albumin (Boyer, Lum, Ballou, Luck & Rice, 1946) and the intensive physicochemical studies of Klotz (1949) have gone far to elucidate both the conditions under which adsorption takes place and the practical importance of the phenomenon (Edsall, 1947). Meanwhile, Scatchard, Scheinberg & Armstrong (1950) and Edsall, Edelhoch, Lontie & Morrison (1950) have shown that serum albumin also binds a number of negatively charged inorganic ions.

The present paper is concerned with a study similar in principle to those previously carried out, of the binding of certain fluorescent dyes to bovine serum albumin. The method used was that developed by Dr G. Weber in this department depending on a measurement of the polarization of the light emitted by excited fluorescent molecules (Weber, 1952).

THEORETICAL

The polarization of the fluorescent light from a dye molecule depends on the relaxation time of rotary Brownian movement of the dye in a given environment and on the time which the dye molecule takes to emit its light following an excitation. If the dye

molecules are able to rotate extensively between excitation and emission, the fluorescent light has little overall polarization while if the molecules rotate very little in this time the emitting oscillators will remain appreciably orientated and will produce an overall polarization of the fluorescent light (Perrin, 1936). For the majority of dyes in aqueous solution the relaxation times of the molecules are small compared with the time taken to emit the fluorescence, and so the polarization of the fluorescence is small (usually less than 1%). The relaxation time may be increased by placing the dye in a viscous medium such as glycerol (Weigert, 1920) or, as Weber has shown, by attaching the dye to a macromolecule such as a protein, which rotates more slowly in aqueous solution than does the free dye. The result of importance in the present study is that the fluorescence of the dye adsorbed on the protein is partially polarized, while the fluorescence of the free dye is usually unpolarized. Therefore, in any mixture of a fluorescent dye with a protein, where a part of the dye is bound to the protein and the remainder is free, the polarization P observed will be less than that for complete binding of the dye P_b depending on the relative contribution which the free and the bound dye make to the total fluorescent intensity. One can say that the polarized fluorescence of the bound dye is diluted by the unpolarized fluorescence of the free dye. A knowledge of the relative intensities of fluorescence of the dye, free and bound, and of the measured polarization of the dye-protein mixture enables the amount of dye bound to be easily calculated as follows. (For derivations of these formulae see Appendix.)

Assuming that the fluorescence of the free dye in solution is unpolarized, the ratio of P to P_b will vary between 0 and 1 as the degree of adsorption of the dye on to the protein is varied. Calling this ratio \bar{p} and supposing the intensity of the fluorescence of the dye-protein system relative to that of the dye alone at the same concentration and pH is R it is shown that the fraction of dye bound (x) is

$$x = \bar{p}R - R + 1. \quad (1)$$

If the dye does not change its fluorescent intensity on adsorption (i.e. $R = 1$) the simple formula

$$x = \bar{p} \quad (1a)$$

* Present address: Postgraduate Medical School, Ducane Rd., London, W. 12.

will hold. On the other hand, if the free dye in aqueous solution possesses a measurable polarization a second modification of Eqn. 1 is needed, namely that, where $\Pi = P_f/P_b$,

$$(1 - \Pi)x = \bar{p}R - R + 1 - \Pi, \quad (1b)$$

and P_f is the polarization of the free dye in solution.

Only one case was found in this work for which the simple formula (1a) could be used (see below); most of the dyes studied showed either a marked increase or a decrease in fluorescence when bound to bovine serum albumin. The significance of these changes in fluorescence will be discussed later, but it is enough to note here that if a marked increase in fluorescent yield occurs on adsorption (as in the case of the sulphonic acid derivatives of 1-naphthylamine), formula (1) ceased to be of practical use since the observed polarization is little diluted by the relatively poor fluorescence of the free dye. In these cases a formula

$$x = \frac{R - 1}{R_{\max.} - 1} \quad (2)$$

has been used, in spite of the fact that this formula does not distinguish between a change in the fraction bound (x) and a redistribution of the already bound molecules among adsorption sites which affect the fluorescence to different degrees. A limitation of this sort is implicit in any indirect method such as this, especially where only one experimental variable is used, and may equally enter into the spectrophotometric technique (Klotz, 1946b). Since formula (1) includes two experimentally determined variables it is less likely to suffer from limitations of this sort and the formula has been used to detect one case (eosin) where the sites are of different kinds.

In order to determine the number of adsorption sites on the albumin and their affinity for the dye the equation derived by Klotz (1946a) was used, namely,

$$\frac{S}{Dx} = \frac{1}{n} + \frac{K}{nD(1-x)}. \quad (3)$$

The known quantities in this equation are S and D , the total protein and dye concentrations respectively, and x , the fraction of dye bound, calculated as described above. The unknown quantities K , the equilibrium constant of dissociation of the eye-protein complex, and n the number of adsorbing sites, may easily be derived from the experimental curves, since n is the reciprocal of the intercept at the axis and K/n the slope of the graph. A greater slope means a more dissociable complex. When the dye concentration is small compared with the protein concentration the simple formula

$$nS \frac{(1-x)}{x} = K \quad (3a)$$

will apply. This is a useful formula since, even if the adsorbing sites differ in combining power, the form of the equation remains the same.

EXPERIMENTAL

Dyes

The dyes used were chosen for their availability and possible experimental value. They can be classified chemically into three classes: (i) sulphonic acid derivatives of 1- and 2-naphthylamine containing one, two or three sulphonic acid groupings; (ii) derivatives of fluorescein and rhodamine with a xanthidrol ring system; (iii) derivatives of acridine. Where necessary these dyes were recrystallized or converted to crystalline derivatives and recovered. The purity of the dyes was examined by chromatography on paper. The term 'dye' has been used for these fluorescent compounds to simplify the nomenclature, although in some cases the absorption maximum of the compounds lies in the near ultraviolet region of the spectrum.

Serum albumin

The protein used was the Armour crystalline bovine serum albumin (batch no. 10522). It was found by comparison with the Armour fraction 5 albumin (batch no. 12 198) that the crystallization had not appreciably altered the adsorbing properties of the protein, but had removed some yellow material whose absorption of the exciting light might complicate the fluorimetric studies. The absorption at the excitation wavelength of 3600 Å. was not great enough in the crystalline product to require any special corrections. The fluorescence of the crystalline protein was also unimportant with the yellow filters (Corning 338, 351, etc.) used for examining fluorescence. The protein was made up in 1% solution, dialysed for 24 hr. at 0° and filtered through Whatman no. 50 filter paper to give a clear solution. The protein concentration was measured in a Zeiss interferometer using the diffusate in the control compartment. The interferometer was calibrated using Kjeldahl nitrogen values for ovalbumin solutions.

Measurement of fluorescence

The protein and dye were mixed and a suitable buffer added. Experiments were made both at constant dye concentration and at constant protein concentration. When a series was carried out at constant dye concentration, only one comparison tube for free dye was required. When the dye concentration was varied, a comparison tube was made up for each dye concentration used. Observations were made in each case on the fluorescent intensity of the dye-protein mixture, as compared with the fluorescence of free dye; and on the polarization of the fluorescence of the dye-protein mixture. In cases where interest was found in the absorption spectrum of the mixture, this was measured in the Beckman spectrophotometer from 6000 Å. down to 3100 Å.

The limiting polarization P_B was measured by so increasing the protein concentration that no further increase in polarization resulted from addition of more protein. This condition was obtained with the dyes at certain pH values where binding was especially strong. The work of Weber (1952) suggests that the same value of P_B would be valid at other pH values.

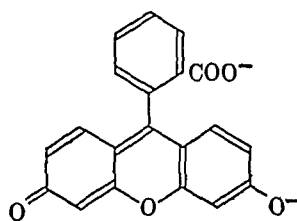
Since polarization measurements are most easily made visually, the intensity measurements were also made visually, the same filters for excitation and observation being used in both cases. Any changes in colour in the

fluorescence during adsorption would affect intensity measurements made with a photocell in an arbitrary way with respect to the eye and make the use of Eqn. 1 more difficult. Fluorescence of wavelength short enough to be readsorbed by the dye was excluded from observation by choice of filters. The visual fluorimeter was made by placing a Pulfrich photometer headpiece vertically at the end of an optical bench and splitting the beam of the usual Siemens mercury lamp, placed on the bench, by means of a modified Pulfrich beam-splitter. The cells containing the fluorescent solutions, size $4 \times 2 \times 1$ cm. were filled completely with the solutions and covered with pieces of optical glass. They were

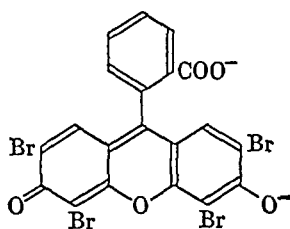
tion of the exciting light (Weber, 1952). In the measurement of polarizations the exciting light was polarized by a Nicol prism in a plane perpendicular to the axis of observation. In the fluorimeter, on the other hand, the exciting light was unpolarized. To make the two sets of measurements comparable the following formula (derived in the Appendix) was used where I' is the observed and I is the corrected intensity:

$$I = \frac{I'}{1 - \frac{1}{2}p}$$

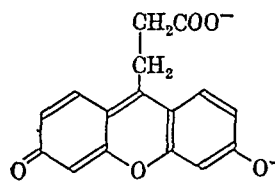
For the theoretical maximum of the polarization of 0.50 this correction is 33 %.



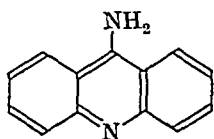
Fluorescein



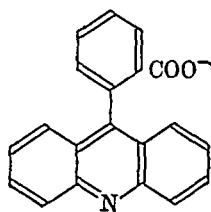
Eosin



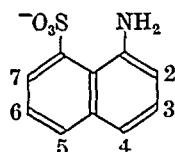
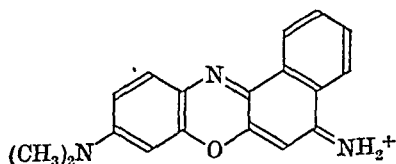
Succinylfluorescein



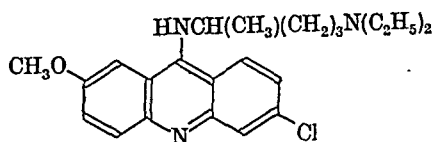
5-Aminoacridine



o-5-Acrydylbenzoic acid

1-Naphthylamine-
8-sulphonic acid

Cresyl fast violet



Mepacrine

Table 1. Combination of fluorescein in borate buffer at pH 9.1

(Fluorescein concentration = 0.13×10^{-5} mol./l.)

Protein concn. ($\times 10^5$ mol./l.) <i>S</i>	Polarization ($\times 100$) $100p$	Relative intensity <i>R</i>	$\bar{p}R$	Fraction of dye bound (<i>x</i>)	Equilibrium constant ($\times 10^5$) $K = nS(1-x)/x^*$
1.46	7.6	0.61	0.13	0.52	1.35 <i>n</i>
2.92	12.0	0.51	0.17	0.66	1.50 <i>n</i>
5.84	17.0	0.37	0.17	0.80	1.45 <i>n</i>

* Where *n* is the number of binding sites.

illuminated on their largest faces and observed through the top. The fluorescent intensities of a series under test were always compared with the brightest fluorescence of the series whether the free dye or the dye combined with the protein.

A simple correction was made to the intensity measurements, since the intensity observed from a solution producing polarized fluorescence is dependent on the polariza-

RESULTS

Table 1 gives some typical results of the combination of the bivalent negative ion of fluorescein with serum albumin in borate buffer at pH 9. The fluorescein concentration was small compared with the protein concentration and so Eqn. 3a applies.

The result of plotting according to Eqn. 3 is a straight line passing through the origin with slope determined by the equilibrium constant.

Table 2 illustrates a second simple case. This is that of the tervalent negative ion of the 2-naphthylamine-3:6:8-trisulphonic acid in unbuffered salt-free protein solution at pH 5.4. In this case there is strong binding to the protein so that over a range of concentration of both protein and dye two molecules of the dye are bound by one molecule of albumin (molecular weight 70 000). Extrapolation from Table 1 shows that fluorescein is only 0.26 times as fluorescent on the protein as in the solution. The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid, on the other hand, is but little altered by adsorption.

Table 2. Combination of 2-naphthylamine-3:6:8-trisulphonic acid at the isoelectric point

Protein concn. $\times 10^5$ mol./l.	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
(a) Dye concentration $= 5 \times 10^{-5}$ mol./l.		
0.60	0.26	2.15
1.21	0.52	2.15
2.42	0.92	1.89

Dye concn. ($\times 10^5$ mol./l.)	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
(b) Protein concentration $= 1.21 \times 10^{-5}$ mol./l.		
5.0	0.50	2.06
10.0	0.258	2.13
20.0	0.132	2.18

Table 3 gives data for the monovalent negative ion of 1-naphthylamine-8-sulphonic acid in phosphate buffer, pH 6.9. In this case the fluorescence of the dye is much increased on the protein (about

Table 3. Combination of 1-naphthylamine-8-sulphonic acid at pH 6.9

(Sulphonic acid concentration 4.15×10^{-5} mol./l.)

Protein concn. ($\times 10^5$ mol./l.)	Relative intensity ($\div 20$)*	Fraction of dye bound (x)	Molecules of dye bound per molecule of protein
0.00	0.05	0.00	—
0.725	0.37	0.34	1.95
1.45	0.62	0.60	1.72
2.90	0.84	0.84	1.19
7.25	1.00	1.00	—

* I.e. relative to the same concentration of free dye, allowing for the increase of fluorescence on adsorption.

20-fold), and the polarization rises to a maximum before the limit is reached in the increase of fluorescence. Using Eqn. 2 it appears that about 2 molecules of this substance are bound to the protein in the concentration range studied.

Effect of pH

As the pH in the surrounding solution is decreased and the charge of protein becomes more positive it would be expected that there would be an increase in the amount of a negative dye bound to

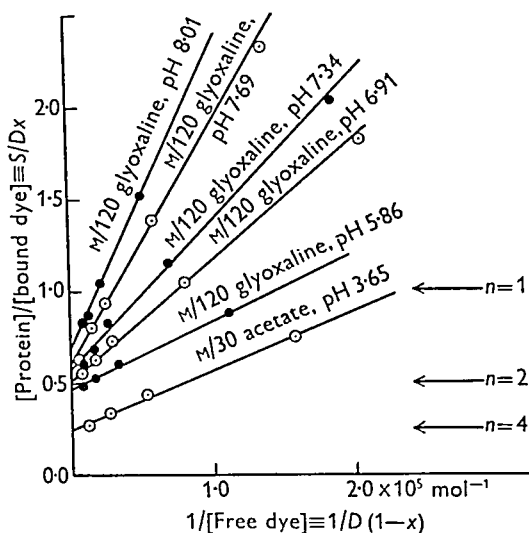


Fig. 1. Adsorption isotherms of 2-naphthylamine-3:6:8-trisulphonic acid.

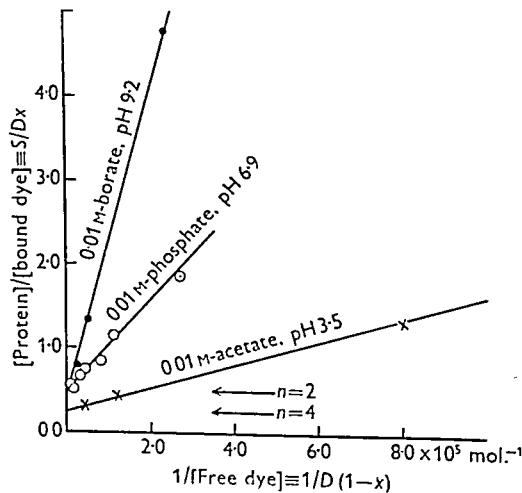


Fig. 2. Adsorption isotherms of fluorescein.

the protein. This is borne out by the following data for 2-naphthylamine-3:6:8-trisulphonic acid (Fig. 1), fluorescein (Fig. 2) and 1-naphthylamine-3:8-disulphonic acid (Fig. 3). It should be noted that fluorescein contains two titratable groups between pH 8 and 4, while the 1-naphthylamine-3:8-disulphonic acid contains one with pK 3.9. These reduce the negative charge on the dye at acid pH relative to alkaline pH.

Figs. 4 and 5 summarize the results obtained by plotting the polarizations obtained at an arbitrary protein concentration against the pH. Fig. 4 is for positive ions (basic dyes) and Fig. 5 is for negative ions (acidic dyes). The results illustrate the familiar observation that an opposite charge on the protein favours the combination of a dye, but there is also

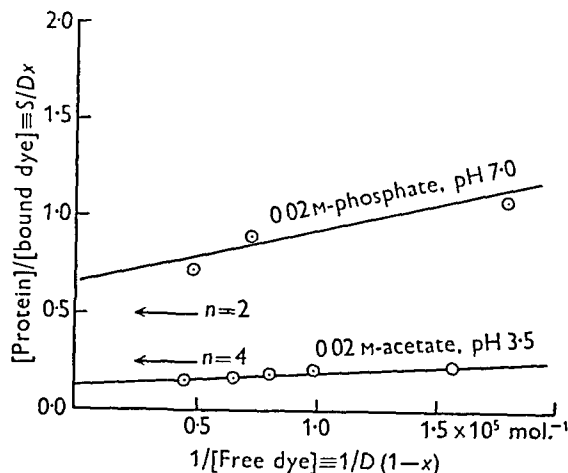


Fig. 3. Adsorption isotherms of 1-naphthylamine-3:8-disulphonic acid.

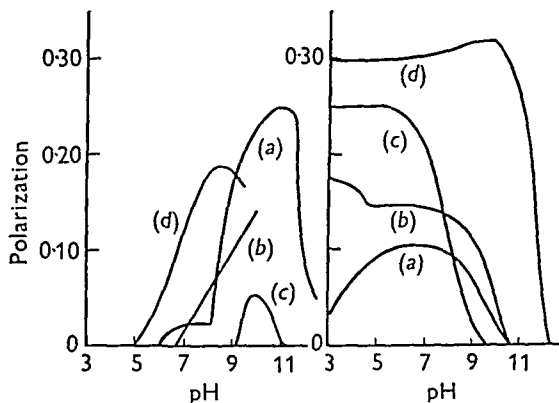


Fig. 4.

Fig. 5.

Fig. 4. Effect of pH on the adsorption of positive dyes (a) mepacrine, (b) cresyl fast violet, (c) 5-aminoacridine, (d) acridylbenzoic acid methyl ester.

Fig. 5. Effect of pH on the adsorption of negative dyes (a) fluorescein, (b) 1-naphthylamine-3:8-disulphonic acid, (c) 2-naphthylamine-3:6:8-trisulphonic acid, (d) eosin.

noticeable the special characteristic of serum albumin. This is apparent in both graphs. In Fig. 5 it is seen that negative ions, even tervalent ions, continue to combine with the protein on the alkaline side of the isoelectric point (pH 5.4). In Fig. 4, though the basic dyes combine in all cases with the protein, there are marked changes in extent of combination at the pK's of certain groups in the dyes. These changes lead to the conclusion that the neutral form of the dye (that form which is more

lipid-soluble) is more strongly adsorbed than the positive ion, even though the average charge on the protein at these pH's would be expected to favour combination with the positive ion.

This is confirmed by the result of adding serum albumin to *o*-5-acridylbenzoic acid at pH 6 where the protein is slightly negative and the dye mostly in the zwitterion form with a negatively charged

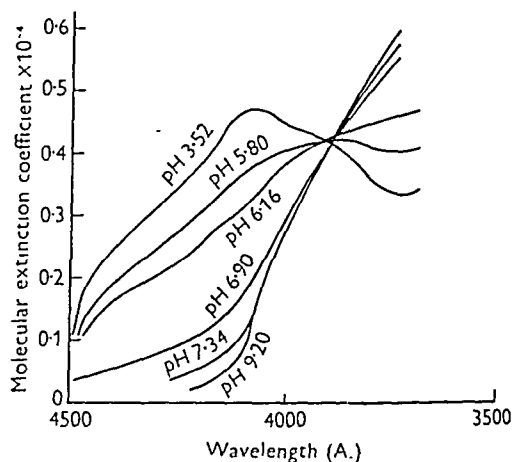


Fig. 6. Effect of pH on the absorption spectrum of *o*-5-acridylbenzoic acid.

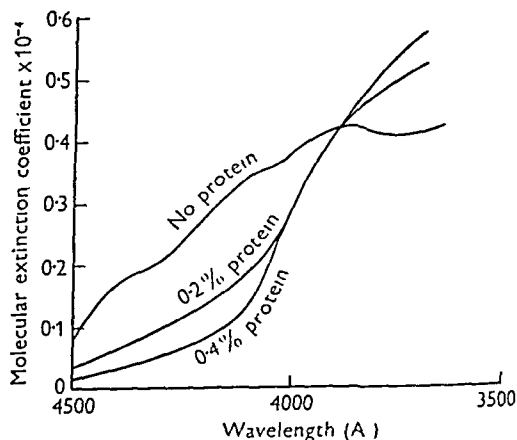


Fig. 7. Effect of the addition of serum albumin on the absorption spectrum of acridylbenzoic acid at pH 6.0.

carboxyl group and a positively charged heterocyclic ring. The serum albumin has the same effect on the dye as does a change of pH toward the alkaline side (Figs. 6 and 7). Addition of serum albumin to the dye at pH 9 when the heterocyclic nitrogen is uncharged has no effect on the spectrum. This 'reversed protein error' can be due to forces like those which produce the other effects discussed here. Fig. 8 shows adsorption isotherms for this dye.

That the neutral and ionic forms of the dyes differ markedly in the P_b values is made unlikely by experiments on the polarization of the fluorescence of the two forms in glycerol-water solutions of various viscosities. The conclusion follows that

while the presence of a charged group on the molecule is not a necessary condition for adsorption to occur, a positive group in contrast with a negative group has a strong inhibitory influence on the combination. This influence extends as far as pH 9.3, the pK of the ring nitrogen in 5-aminoacridine (Albert & Goldacre, 1943) when the protein has a net charge of about -30 (Tanford, 1950).

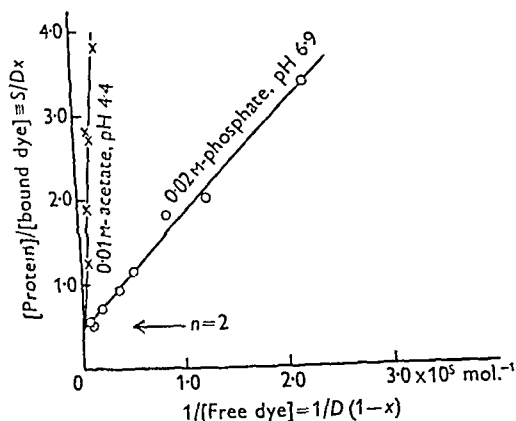


Fig. 8. Adsorption isotherms of acridylbenzoic acid.

Effect of modifications in structure of the dye

Some results of interest to the general theory of adsorption are obtained when the effect of minor alterations in the structure of the dye on its adsorption properties is studied. Fig. 9 shows results with fluorescein as compared with eosin in neutral phosphate buffer. Eosin is a case where the intensity does not change linearly with the calculated fraction bound (x) but first decreases and then increases as the protein concentration is increased. This unusual effect can be explained if only a few of the total binding sites yield an appreciably fluorescent adsorption complex and if it is assumed that these are the most actively combining sites. While the dye is in excess of the total sites, both the sites of low and of high fluorescent yield will be occupied. If an excess of sites is present, however, the sites of higher fluorescent yield will be favoured and the fluorescent intensity will increase again. There is therefore a minimum of fluorescence depending on the ratio of dye to protein. Using the formula 1b the amount bound is found to increase continuously over the range of protein concentration.

The naphthylaminesulphonic acids tried, namely the 1-naphthylamine-5- and 8-monosulphonic acids, the 3:8-disulphonic acid and the 3:6:8- and 4:6:8-trisulphonic acids and the 2-naphthylamine-3:6:8-sulphonic acid, showed binding of about the same extent at neutral pH. Methylation of the amino group increases the extent of combination of the 1-naphthylamine-5-sulphonic acid with serum albumin.

It can also be shown that fluorescein methyl ester and *o*-5-acridyl-benzoic acid methyl ester are adsorbed to about the same extent as the free acids. Sulphofluorescein is adsorbed similarly to fluorescein, and succinylfluorescein, with a short side chain instead of a ring, less than fluorescein.

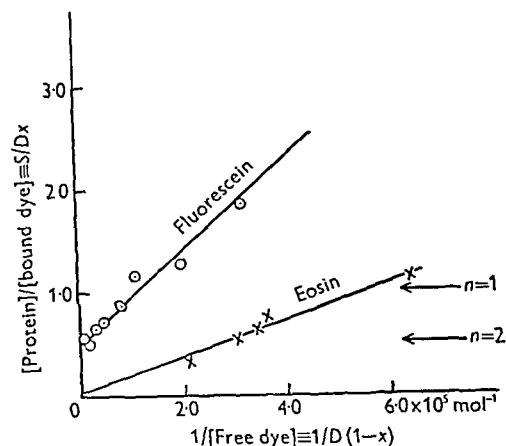


Fig. 9. Adsorption isotherms of eosin and fluorescein in 0.01 M-phosphate pH 6.9.

Competition experiments

As it has so far been described, the method of the polarization of fluorescence is limited to studying the combination of fluorescent substances only. A knowledge of the ability of various substances to compete with suitable test dyes leads, however, to an extension of the method in a more indirect form (Klotz, 1949). There are two easy ways of carrying out competition experiments:

(i) With a given quantity of serum albumin and of test dye the polarization P_1 is recorded and, in addition, the polarization P_2 (always less than P_1) which results when a known further amount of the same dye is added. If, instead of this further addition, an equimolar amount of a non-fluorescent competitor is added and a polarization P_3 results we have either

$P_3 = P_1$	The substance shows no competition
$P_3 = P_2$	Competes as strongly as the test substance
$P_1 > P_3 > P_2$	Competes less than the test substance
$P_3 < P_2$	Competes more than the test substance

This is illustrated by Table 4, where various derivatives of naphthalene are used to compete with the 2-naphthylamine-3:6:8-sulphonic acid. The sulphonic groups, but not the amino groups, seem necessary for competition. As the 1- and 2-naphthylamines themselves have no effect on the polarization, it seems likely that they do not combine at the site of combination of the test substance at the concentration used.

(ii) For substances whose combination with protein is fairly weak, competition can be studied by adding the competitor in great excess relative to the protein and noting the decrease in the polarization.

Table 4. *Competition of related compounds with 2-naphthylamine-3:6:8-trisulphonic acid (pH 6.7)*

Competitor	Amount of test substance (ml.)	Amount of competitor (ml. equimolar solution)	Polarization
None	1.0	—	0.060 P_2
None	0.2	—	0.116 P_1
1-Naphthylamine	0.2	0.8	0.115 P_3
2-Naphthylamine	0.2	0.8	0.117 P_3
Naphthalene-1-sulphonic acid	0.2	0.8	0.065 P_3
Naphthalene-2-sulphonic acid	0.2	0.8	0.052 P_3
Naphthalene-3:6:8-trisulphonic acid	0.2	0.8	0.035 P_3
<i>N</i> -Acetyl-1-naphthylamine-8-sulphonic acid	0.2	0.8	0.071 P_3

The present experiments have been confined to comparing the relative affinities of related simple substances as revealed by this method, although an absolute derivation should be possible.

Table 5 gives data for the ability of various ions to compete at the same molar concentrations, again using the 2-naphthylamine-3:6:8-trisulphonic acid as test substance. The outstanding trend in this table is that whereas alteration of the cation has little or no effect on the extent of the combination the ability of different anions to compete is markedly different. This agrees with the results found both in this study and by other workers that preferential combination occurs with the negative ion of an ionic compound in solution.

The competition with the series of univalent anions $F^- < Cl^- < Br^- < NO_3^- < I^-$ lies in their order in the so-called Hofmeister series relating to gelatin (Loeb, 1924) and this shows that the simple assumptions of the Debye-Hückel theory cannot in this case be sufficient to explain the effect of these salts. The effect of bromine atoms (see results with eosin above) and of nitro groups (Teresi & Luck, 1948) in organic molecules seem to be paralleled by the strong binding of the nitrate and bromide ions.

Table 6 gives a similar set of figures for some organic anions and one cation. The differences observed between the different compounds in this

Table 5. *Competition of some ions with 2-naphthylamine-3:6:8-trisulphonic acid (pH 6.7)*

Competitor (M/15):	Polarization
None	0.202
NaCl	0.100
KCl	0.100
Na_2SO_4	0.015
K_2SO_4	0.015
Sodium acetate	0.130
Sodium succinate	0.015
Sodium benzoate	0.000
NH_4Cl	0.100
$CsCl$	0.101
NaF	0.181
KCl	0.098
KBr	0.050
KI	0.000
NaN_3	0.045
$NaNO_3$	0.015
Competitor (M/30):	
K_2SO_4	0.045
$(NH_4)_2SO_4$	0.047
Li_2SO_4	0.048
$MgSO_4$	0.070

table are of importance for the theory of adsorption discussed below.

The 2-naphthylamine-3:6:8-trisulphonic acid was used in these studies because its fluorescence intensity is little changed on adsorption and its

Table 6. *Competition of organic ions with 2-naphthylamine-3:6:8-trisulphonic acid (pH 6.7)*

Competitor	Competitor concn. ($M \times 10^{-2}$)				
	0	1	1.5	3.5	6
	Polarization				
Sodium galacturonate	0.205	0.205	0.205	0.205	0.205
Sodium phenylacetate	0.205	0.162	0.102	0.000	0
Sodium cyclohexylacetate	0.205	0.071	0	0	0
Sodium caprylate	0.205	0	0	0	0
Octylamine hydrochloride	0.205	0.205	0.205	0.148	0.095*
	Competitor concn. ($M \times 10^{-3}$)				
	0	1	1.5	2.5	
	Polarization				
Sodium caprylate	0.208	0.140	0.067	0	

* Attributable to the chloride ion alone.

polarization has, therefore, a linear relationship to the fraction which is bound. The next section considers more generally the changes in fluorescent properties and absorption spectra of the dyes.

Change in fluorescence and absorption spectra on adsorption

In the previous sections the change in fluorescent intensity on adsorption has been mentioned as an important factor to be considered in applying the method of the polarization of fluorescence to a study of adsorption isotherms. During adsorption there are also changes in the absorption spectra of the dyes. The changes may be summarized conveniently according to the chemical types of the dye molecules.

(i) *Sulphonic acid derivatives of 1- and 2-naphthylamine.* The fluorescence of the 2-naphthylamine-3:6:8-trisulphonic acid is bright sky blue and is slightly quenched when the dye is on the protein without a change in colour. The fluorescences of the 1-naphthylamine derivatives are greenish yellow in aqueous solution and this is also the case with the dimethylaminonaphthalene derivative. The fluorescence of all these compounds is increased when they are adsorbed on the protein and in most cases it is changed to the bright blue of the 2-naphthylamine compound described above. This change is most marked with the relatively weak fluorescence of the 1-naphthylamine-8-sulphonic acid where a 20-fold increase in brightness occurs. With the stronger fluorescence of the others in this series, increases of two or fourfold are observed. The change may be due to an increase in the dipole moment of the amino group on adsorption, as it is known that 2-naphthylamine possesses a higher dipole moment than 1-naphthylamine (Bergmann & Weizmann, 1936). Shifts to the red of 80 Å. were observed in the ultraviolet spectra of the 1-naphthylamine compounds when adsorbed.

(ii) *Xanthidrol derivatives.* The fluoresceins and rhodamines tried were all quenched on the protein. For fluorescein itself the intensity was 0.26 of that of the free dye. In addition, characteristic shifts in the absorption spectrum towards the red occurred. This effect was most marked with the methyl ester of fluorescein where the sharp absorption bands of the free and of the adsorbed dyes are clearly separated at the maxima, while the shape and height of the curves are hardly altered (Fig. 10). The colours of solutions of the adsorbed methyl ester are bright pink while that of the free dye at alkaline pH is orange yellow. This is not an effect of pH, as the absorption of the different ionic forms of fluorescein moves progressively towards the blue with increased acidity. In the case of eosin the adsorbed dye has a moderate adsorption at the green line of mercury, namely, 5460 Å. while the free dye absorbs little at

this wavelength. The tubes which contain adsorbed material can therefore be distinguished easily as they are brightly fluorescent in this light, whereas tubes with the free dye alone are by comparison practically non-fluorescent.

(iii) *Acridine derivatives.* Considerable variation was found in the behaviour of the fluorescences of the acridines when adsorbed. The fluorescence of *o*-5-acridylbenzoic acid was quenched to one-tenth of its value in solution without change in its absorption spectrum. The fluorescence of mepacrine increased slightly.

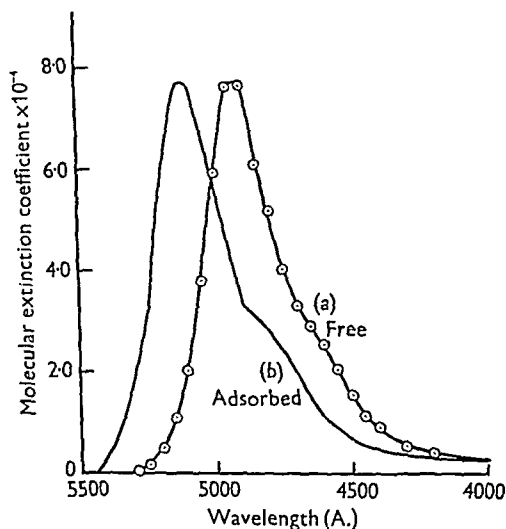


Fig. 10. Absorption spectrum of fluorescein methyl ester at pH 9 (a) free dye, (b) dye adsorbed on serum albumin or cetyltrimethylammonium bromide.

The relation of the changes in fluorescences and absorption spectra to the state of the environment of the dyes

Since the change in the fluorescences and the absorption spectra of the dyes is likely to be characteristic of some new electronic state due to an environment at the site of adsorption different from that in water, it was considered likely that a simulation of these changes in simple physico-chemical systems would lead to an understanding of the nature of the adsorbing region on the protein. A suitable series of dyes was chosen to test this, namely, eosin, fluorescein methyl ester, 1-dimethylaminonaphthalene-5-sulphonic acid, 1-naphthylamine-8-sulphonic acid and *o*-5-acridylbenzoic acid. By trial it was found that the kationic detergent, cetyltrimethylammonium bromide (CTAB), gave reactions with these dyes very similar to those given by bovine serum albumin. That adsorption by the detergent micelles had occurred was demonstrated by showing that the detergent in low concentration increased the polarization of eosin excited with the

green line of the mercury lamp and of fluorescein excited with the blue lines. Klotz (1947), using the azo-dye methyl orange, has shown a similar parallel between the effect of protein and of CTAB on the absorption spectrum. The ring structure of the dye which acts as the oscillator (Benel, Kastler & Rousset, 1940) is likely to be in the micelle rather than adsorbed on its surface (Hartley, 1937), and hence in a substantially organic environment free from water dipoles.

An important property of the interior of the micelle is its low dielectric constant, and in a single-phase system the dielectric constant of the solutions of the dyes was lowered by adding dioxan (Akerlof & Oliver, 1936) which had been purified by the

vents of non-polar nature. Glycerol, concentrated sugar solution and ethanol gave smaller shifts to the red with respect to water, although the refractive indices and hence the volume-polarizabilities of these liquids was as high or higher than the benzene and 'essence'. A similar view is admitted by Seshan (1936).

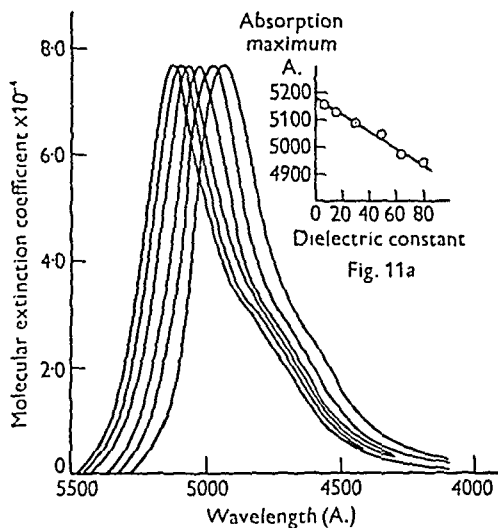


Fig. 11. Effect of dioxan concentration on the absorption spectrum of fluorescein methyl ester (from right to left, increasing dioxan concentrations from 0 to 90%.)

Fig. 11a. Shift in the wavelength of the absorption maximum with increasing dielectric constant of the dioxan-water mixtures.

method of Oxford (1934). The dyes behaved in a way similar to that observed when they were adsorbed by serum albumin and CTAB, the most striking similarities being shown by fluorescein methyl ester, 1-naphthylamine-8-sulphonic acid and acridylbenzoic acid (Figs. 10-13). The effect of solvents of low dielectric constant in imitating the effect of serum albumin is such that we could almost without exception predict how a given dye would change its fluorescence and spectrum from its behaviour in dioxan solution. Evidence that the dielectric constant of the medium is the determining factor in the changes in spectrum of the xanthodrol compounds may also be obtained from the results of Vaillant (1927) for erythrosin, though this author does not interpret his results in this way. He found that the largest shifts to the red in the spectrum of erythrosin were in benzene and 'essence', both sol-

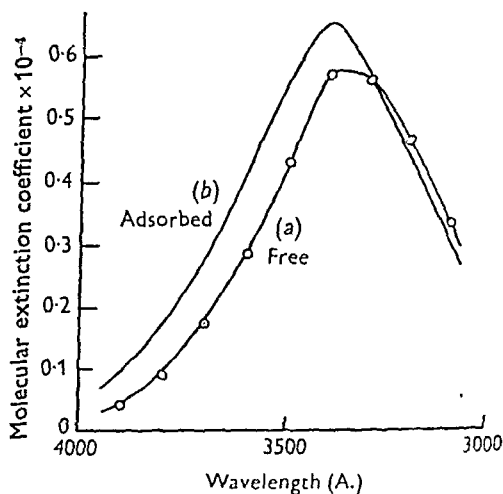
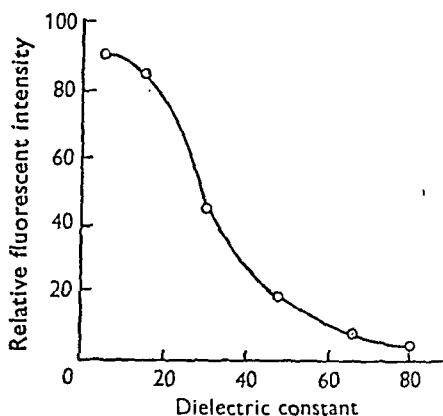


Fig. 12. Effect of dioxan on the absorption spectrum and fluorescent intensity of 1-naphthylamine-8-sulphonic acid. (a) spectrum of dye in aqueous solution, (b) spectrum of dye adsorbed on serum albumin or in 90% dioxan solution.

It may be concluded that the behaviour of the fluorescent dyes when adsorbed on to serum albumin identifies the site of adsorption with material of low dielectric constant. The use of a series of these dyes should enable other systems of amphipathic behaviour to be easily distinguished as has already been stated (Sheppard & Geddes, 1945).

Relation to the work of Dr G. Weber with fluorescent groups coupled by chemical reaction with proteins

Of the dyes used in this paper, two, namely the 2-naphthylamine-3:6:8-trisulphonic acid and the 1-dimethylaminonaphthalene-5-sulphonic acid, have

been used by Dr G. Weber as compounds suitable for coupling with proteins by covalent bonding. It is possible to carry out studies using serum albumin similar to those of Weber (1952) by ensuring that all the dye is combined with protein and plotting a curve of $1/P_B$ against T/η , where T is the absolute

eosin excited by Hg 5460 Å, where an extrapolation of $1/P_B$ to $T/\eta = 0$ gives the accepted value for eosin in very viscous solution. The colour of the fluorescence of the serum albumin-sulphonamide conjugate is greenish blue like the colour of fluorescence of the simple sulphonamides in 90% dioxan. Digestion of the albumin conjugate with Armour crystalline pepsin results in the fluorescence becoming orange like that of the sulphonamides in water. Even with native proteins the greenish-blue fluorescence of albumin conjugates is not universal (Weber, 1952) and the yellowing of the fluorescence of the sulphonamides as the polarity of the medium is increased may provide a convenient yardstick against which the polarity of the protein surface in the neighbourhood of the primary amino groups may be estimated.

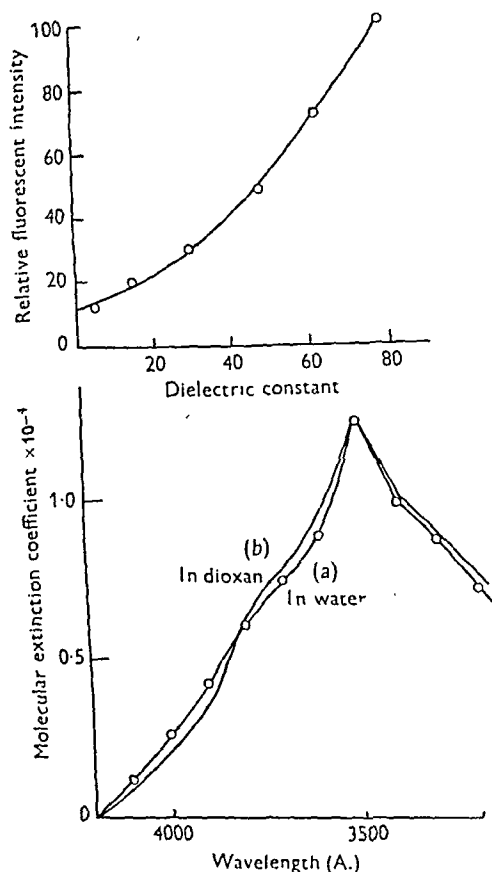


Fig. 13. Effect of dioxan on the absorption spectrum and fluorescent intensity of *o*-5-acridylbenzoic acid. (a) spectrum of dye in aqueous solution, (b) spectrum of dye in 90% dioxan.

temperature and η the viscosity. The intercept of this line with the ordinate gives the value of $1/P_B$ in the absence of Brownian movement, and the slope is determined by the lifetime of the excited state and by the size of the protein. In either case the value of $1/P_B$ was found to be the same at $T/\eta = 0$ for the coupled as for the adsorbed dye. The slope of the graph for the adsorbed 1-dimethylaminonaphthalene-5-sulphonic acid was found to be twice that for the coupled sulphonamide conjugate (Fig. 14). Since Weber (unpublished observations) has shown that the lifetime of excited state of the acid in glycerol is about twice that of the derived sulphonamide, these results show that the adsorbed dye has a fixity in the albumin surface comparable with that of the coupled dye. A similar result is obtained for

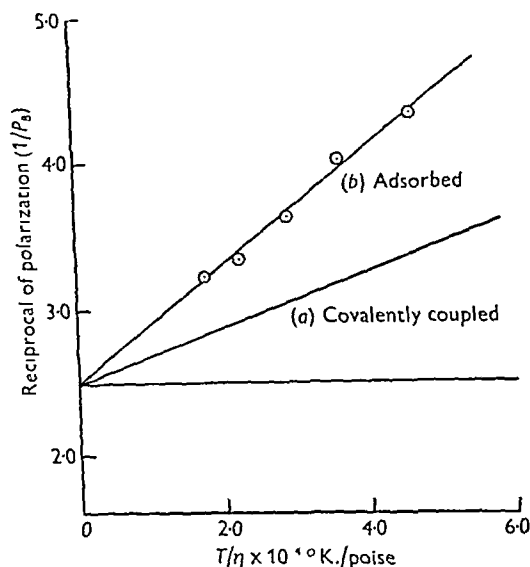


Fig. 14. Plot of polarization at different temperatures according to the Perrin equation for the 1-dimethylaminonaphthalene-5-sulphonic acid attached to serum albumin (a) covalently coupled (Weber's data), (b) adsorbed.

DISCUSSION

The evidence presented here and that obtained by other workers leads to the view that there are several kinds of physicochemical mechanism operating in the process of adsorption on to serum albumin.

It is clear that the charge on the molecule is of importance, negative ions being adsorbed in preference to positive ions at the sites of adsorption. Superimposed on this is a variation in binding depending on the nature of the ion. It is often supposed that this effect is due to van der Waals forces between the protein and the adsorbed molecule. Since, however, for compounds of about the same molecular weight, namely, caprylic acid, cyclohexyl

acetic acid and phenyl acetic acid, one obtains different degrees of binding with the benzene moiety less active than the paraffin moieties, this does not seem to be an adequate hypothesis.

Any organic molecule may be considered as an assembly of polar and of non-polar parts, and in aqueous solution the relation of these parts with the water will be very different. Non-polar material introduced into water produces a separation of the strongly bonded water molecules and replaces the interaction of water molecules with each other by a weaker interaction of water with the non-polar substance. Reversal of this process with recombination of the separated molecules of water results in a liberation of energy which corresponds to the energy of the surface of the non-polar material in solution. The polar parts of the molecule, in contrast, can associate with the water as well as can water with itself (Langmuir, 1925) and the adsorption of the non-polar part of the organic molecule on to or into a non-polar surface, leaving the polar parts in the aqueous phase, is therefore accompanied by a liberation of energy, since the surface of the non-polar material in contact with the water is decreased.

A semi-quantitative way of stating this is to assign to water a dipole W and to the so-called non-polar material a smaller dipole P (Gent, 1948). Before adsorption the dipole interaction energy is proportional to $2PW$ and after adsorption to $P^2 + W^2$. The change in energy is $P^2 + W^2 - 2PW$ or $(W - P)^2$ which increases as the difference between the respective dipoles increases and is always positive.

From the behaviour of the fluorescent dyes when adsorbed there is good reason to suppose that adsorption does remove the dye from the water to a second medium of lower dielectric constant, and so it seems plausible to attribute the adsorption in great part to a rejection from the water structure rather than an attraction by the protein. The concept of surface energy further accounts for the relatively low activity of a benzene moiety as compared with a paraffin, since the surface energy of benzene in water is less (34 ergs/cm.²) than for a paraffin (54 ergs/cm.²); benzene is more hydrophilic than paraffin. As would be expected, galacturonic acid, although its molecular weight is higher than most of the simple competitors included in Table 6, is ineffective in competition, as it is strongly hydrophilic and the force of rejection is weak.

In terms of the serum albumin molecule the amino-acid composition of the protein is adequate to provide the sort of surface against which these forces can act. It is visualized that the region of adsorption is one where a local excess of positive groupings is backed by a local excess of the hydro-

phobic side chains of leucine, valine and other non-polar amino-acid residues. Because of the low dielectric constant of this microphase the local charge is shielded from the general field of the protein and is not as greatly affected as it would otherwise be by variations in the net charge of the protein as a whole (Hartley, 1938). To account for the absence of corresponding sites of adsorption for positive ions, it must be supposed that the negative groups on the protein are more diffusely arranged and lack the hydrophobic backing which the positive groups have.

For each molecule the extent of adsorption will depend both on non-polar forces and attraction of negative ions. In the series of naphthylamine-sulphonic acids, for example, the addition of a sulphonic group will decrease the former and increase the latter type of interaction, the effect near the isoelectric point being to keep the total binding of these acids appreciably constant. An additional type of force may be important with bromine, iodine and the nitro group, both in inorganic ions and in organic binding. This could be a polarization in the field between the protein and the orientated dipoles of water. The low activity of the fluoride ion, as compared with the chloride, may be due to its increased hydration, keeping it in the aqueous phase by means of hydrogen bonding.

In a recent review Klotz (1949) has described his views on the special structure of the serum albumin molecule and has criticized the ideas of Davis (1943). The present work supports and extends the ideas of Davis and is in disagreement with the views of Klotz, as it underlines the importance of the surface active properties of those amino-acids residues usually regarded as inert, namely, the leucines, valine, etc. Klotz, in contrast, believes the stoichiometric relations of hydroxy amino-acids with the dicarboxylic acids to be of paramount importance. The correlations given by Klotz are nevertheless suggestive, as it is for those proteins where the hydroxyl groups are relatively less numerous than the hydrophobic backing of charged groups would be most easily achieved and the forces described in this paper most likely to be active. In view of the non-polar nature of the active surfaces on the protein and of the adsorbed molecule the idea of 'combined' water molecules being displaced on adsorption (Klotz, 1949) would seem unlikely.

The results with the dyes used in this work show that about 2-4 molecules of dye are adsorbed by each molecule of the protein at neutral pH, with the exception of eosin where more molecules combine. A decrease in the pH increases the number of sites available to negative dyes. Rosenfeld & Surgenor (1950) have found 2 molecules of ferriprotoporphyrin strongly bound to the human serum albumin molecule, while Martin (1949) has found 3 molecules

of bilirubin bound per molecule of albumin. These results contrast with the much higher values from the dialysis experiments of Klotz (1946a) and other workers, e.g. Teresi & Luck (1948) (10–25 molecules bound per albumin molecule). Klotz (1946b) has never reported these high values as a result of his spectrophotometric technique, and it may be that the dialysis technique when applied to higher dye concentration includes some type of interaction between the dye and the protein unlike that occurring at lower concentrations and measured by the present method. Certainly the low values are more in accord with the view that the effect of the positive groups is reinforced as described in this paper in a few special patches on the protein, while the higher values accord better with the view of Klotz that the amino groups need only to be freed from hydrogen bonding with other amino-acid residues in order to be active. The results of a comparison with Weber's work on covalently coupled dyes, discussed above, strongly support the idea of a special patch of positive groupings as opposed to a general activation.

Whether a similar active region is important in cases of interactions of a more specific nature requires further evidence to decide. It is likely that the forces related to surface tension described here play a part in all interactions of non-polar material in aqueous solution, and the forces of van der Waals should therefore not be used indiscriminately to account for these interactions. The analogy between the electronic state of the dye molecules at the protein surface and in organic media should be borne in mind when attempting to formulate a theory of enzyme activation.

SUMMARY

1. A method is described for studying quantitatively the adsorption of fluorescent dyes by macromolecules using measurements of the polarization and intensity of the fluorescent light. The method is applied to the adsorption of dyes by bovine serum albumin.

2. The effect of pH on the adsorption is discussed. Negatively charged dye ions are adsorbed even when the net charge on the protein is negative. A positive charge on the dye molecule inhibits combination on either side of the isoelectric point. Basic dyes are adsorbed as the free base but not as the positive ion.

3. Free carboxyl, sulphonic, hydroxyl or amino groups in the dye are not necessary for combination between dye and protein to occur. Bromine atoms increase the extent of adsorption.

4. Addition of related but non-fluorescent compounds can decrease the adsorption of a fluorescent dye by competition. Competition is also found between the dye and a variety of simple inorganic and organic ions. The activity of univalent negative inorganic ions is in the order of the Hofmeister series and that of univalent negative organic ions in the order of surface activity. Positive ions do not compete with the dyes.

5. Characteristic changes in the absorption spectra and in the fluorescence of the dyes occur on adsorption. These changes can be reproduced if the dyes are adsorbed by cetyltrimethylammonium bromide micelles or dissolved in 90 % dioxan.

6. The significance of these results is discussed and a comparison made between the adsorbed dye and the dye coupled covalently to serum albumin by the methods of Dr G. Weber. It is concluded that the results of this work can best be explained by the presence in serum albumin of a few regions of surface where a local excess of positively charged amino-acid residues is backed by residues with non-polar side chains.

I am indebted to Dr G. Weber of this laboratory for initiating this work, for critical advice during its progress, and for making available the apparatus for measurement of polarization of fluorescence and many of the organic chemicals used. I would also like to thank Dr M. R. J. Sultan of the Department of Colloid Science, Cambridge, for providing the cetyltrimethylammonium bromide, Dr F. A. Isherwood of the Low Temperature Research Station, Cambridge, for a gift of purified galacturonic acid and Messrs Imperial Chemical Industries Ltd. for the naphthylaminesulphonic acids. The work was carried out during the tenure of a grant from the Medical Research Council.

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APPENDIX

Derivation of equations

Formulae (1) are derived as follows: consider a solution of dye with fluorescent intensity I_0 and polarization P_F to which protein is added. As the concentration of protein increases the fraction of dye bound to the protein increases. A small addition of protein will remove an amount of dye δx , of fluorescent intensity $I_0 \delta x$, from solution. Attached to the protein this amount of dye will have a different intensity $\lambda_x I_0 \delta x$, say, where λ_x may vary during the course of a large addition of protein. It will also have a polarization P_B larger than P_F . When a fraction x of the dye is bound the intensity of the mixture I will be

$$I = I_0 - \int_0^x I_0 dx + \int_0^x \lambda_x I_0 dx$$

$$= I_0 \left(1 - x + \int_0^x \lambda_x dx \right). \quad (i)$$

The polarization p is the average for free and bound dye weighted according to their respective intensities, i.e.

$$p = \frac{(1-x) P_F + \int_0^x P_B \lambda_x dx}{1-x + \int_0^x \lambda_x dx}. \quad (ii)$$

With P_B not dependent on x , the integral $\int_0^x \lambda_x dx$ is eliminated from (ii) using (i). Calling

$$P/P_B = \bar{p}, \quad P_F/P_B = \Pi, \quad I/I_0 = R;$$

$$\bar{p} = \frac{(1-x) \Pi + R + x - 1}{R}, \quad (iii)$$

$$\text{or} \quad (1 - \Pi) x = \bar{p} R - R + 1 - \Pi. \quad (1b)$$

Equations (1) and (1a) are derived from this by putting $P_F = \Pi = 0$ and $R = 1$ progressively.

Formula (2) is based on a simple proportionality argument. Formula (4) is derived as follows: Suppose the fluorescence is excited by light plane polarized at an angle θ to the axis of observation and has a polarization p . The observed intensity I' will be the sum of three components, namely,

$$I' = I_1 \sin^2 \theta + I_2 \cos^2 \theta + I_2, \quad (iv)$$

where I_1 and I_2 are the intensities of the components of fluorescence parallel and perpendicular to the exciting vector and so $p = \frac{I_1 - I_2}{I_1 + I_2}$ by definition, or $I_2 = \frac{(1-p)}{(1+p)} I_1$. Unpolarized excitation is equivalent to averaging for all angles θ or since $\overline{\sin^2 \theta} = \overline{\cos^2 \theta} = \frac{1}{2}$;

$$I' = \frac{1}{2} I_1 + \frac{3}{2} I_2$$

$$= I_1 \left(\frac{1}{2} + \frac{3}{2} \frac{1-p}{1+p} \right)$$

$$= I_1 \left(\frac{2-p}{1+p} \right). \quad (v)$$

For excitation perpendicular to the direction of observation on the other hand $\theta = 90^\circ$, and

$$I = I_1 + I_2$$

$$= I_1 \left(1 + \frac{1-p}{1+p} \right)$$

$$= I_1 \left(\frac{2}{1+p} \right). \quad (vi)$$

Combining (v) and (vi)

$$I = \frac{I'}{1 - \frac{1}{2}p}. \quad (4)$$

Enzymes of the Potato Concerned in the Synthesis of Starch

1. THE SEPARATION AND CRYSTALLIZATION OF Q-ENZYME

BY G. A. GILBERT AND A. D. PATRICK

Department of Chemistry, The University, Edgbaston, Birmingham

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Following an early recognition that plant starches are not homogeneous, extensive work in the last decade has established that most plant starches contain two main components. The isolation and characterization of the components is reviewed fully elsewhere (Kerr, 1950) and need not be described here. It is sufficient to say that these components, for which the names amylose and amylopectin are becoming commonly accepted, are readily separated from each other by fractional precipitation from aqueous solution with organic reagents, and that this difference in solubility and in other physical properties is due not to any considerable difference in chemical composition, but to a difference in molecular shape. Thus both components are polymers of glucose of high molecular weight, but amylose has a linear, or at most very slightly branched structure, while amylopectin has a very considerably branched structure.

Amylose is composed essentially of a single chain of glucose units joined by α -1:4 bonds. The exact structure of amylopectin is not known, but the available evidence (Halsall, Hirst, Hough & Jones, 1949) is consistent with the view of Haworth, Hirst & Isherwood (1937) of a main chain of glucose units linked as in amylose by α -1:4 bonds, but with an average of about every twelfth such bond replaced by an α -1:6 bond. At each of these positions a side chain of about twelve α -1:4 linked glucose units is attached at its reducing end by an α -1:4 bond. Half of the amylopectin molecule is therefore in the form of short side chains.

Along with the elucidation of the composition of starch, some insight was gained into the biological origin of its components: amylose was shown by Hanes (1940*a, b*) to be formed from glucose-1-phosphate by the action of the plant enzyme phosphorylase; amylopectin was shown by Haworth, Peat & Bourne (1944) to be formed from amylose by the plant enzyme, Q-enzyme.

The synthetic polysaccharides prepared in the laboratory with these enzymes are likely to prove of assistance in the study of natural starch, and, in addition, they can serve as model substances for the study of the effect of degree of branching on the properties of polymers. Such studies require the

provision of pure enzymes, and it was the purpose of the present work to obtain phosphorylase and Q-enzyme of a sufficient degree of purity.

Part of the method adopted was based on the technique devised at the Harvard Medical School, Boston, for the fractionation of blood plasma. In this technique precipitation is carried out at low temperature, with ethanol, from solutions of sufficiently low ionic strength for the specific properties of the proteins to exert their full effect (Cohn *et al.* 1946; Cohn *et al.* 1950). The separation of Q-enzyme by these methods, reported briefly earlier (Gilbert & Patrick, 1950), is described in this paper, and the separation of phosphorylase is described in the following paper.

METHODS

Preparation of crystalline Q-enzyme. Autumn harvested, non-sprouting King Edward potatoes were peeled carefully, washed with tap water and sliced. The slices (400 g.), after steeping for 0.5 hr. in 500 ml. of $\text{Na}_2\text{S}_2\text{O}_4$ solution (7 g./l.) were drained, washed thoroughly with tap water, and minced. The juice was pressed from the pulp through fine muslin by a hand press, and clarified by centrifuging. About 100 ml. were usually obtained. Substitution of a blender (at 0°) for a mincer improves the yield of juice to about 200 ml. From this point all operations were carried out in a cold room set at -5°, or in a refrigerated centrifuge. A 50% (v/v) solution of ethanol containing 0.01 M-disodium hydrogen citrate was prepared for use as a precipitant (50 ml. ethanol, 53.7 ml. water). Its pH was adjusted with NaOH until a pH of 6.0 at 20° was recorded for a portion diluted with 5 times its volume of 0.01 M-NaCl. The precipitant was cooled to -5°, and added at a maximum rate of 25 ml./min. with thorough but gentle stirring. A further 15 min. were allowed for equilibration before centrifuging. The temperature of the solution to which the precipitant was being added was kept about 1° above its freezing point by a refrigerator bath of ethanol.

The potato juice (100 ml., pH 5.8-6.0) was cooled to 0°, avoiding freezing, and 28.2 ml. of 50% ethanol added to bring the concentration of ethanol to 11% (at a final temperature of about -2°). After centrifuging the solution at -2° the precipitate was discarded and the ethanol concentration of the supernatant raised to 14.9% by adding 0.11 ml. of 50% ethanol/ml. of supernatant to give precipitate I. (The figure 16 instead of 14.9 was given in error by Gilbert & Patrick (1950).) The precipitate was separated by centrifugation at -5°, dissolved at 0° in 60 ml. of citrate

buffer (0.01 M-disodium hydrogen citrate, NaOH to pH 6.0), and 16 ml. of 50% ethanol added to give a concentration of 10.5% (v/v). The resulting precipitate was discarded, and the concentration of ethanol raised to 15% by the addition of 0.11 ml. of 50% ethanol/ml. of supernatant as before. Precipitate II which formed was dissolved at 0° in 40 ml. of 0.01 M-citrate solution, pH 6.0, and reprecipitated at -5° with 18.9 ml. of 50% ethanol to give precipitate III. This was dissolved at 0° in 5 ml. 0.05 M-citrate buffer pH 7.0 and the solution freeze-dried to enable the experiment to be interrupted at this point. The dried material was later reconstituted with 20 ml. water and the ionic strength of the solution increased 3.6 units by the addition of 9.3 ml. of 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution (50 g./100 ml. solution, NH_3 to pH 6.2). After standing for 2 hr. at 0° with frequent stirring, the solution was centrifuged at 0° and precipitate IV removed.

Precipitate IV was dissolved at 0° in 10 ml. of $(\text{NH}_4)_2\text{SO}_4$ solution (ionic strength 1.0); (4.4 g./100 ml., NH_3 to pH 6.2), and the solution filtered first through a Whatman no. 42 paper under gravity, and then through a sintered-glass filter (grade 3) under slight positive pressure. After raising the temperature to 10°, 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution pH 6.2 (filtered through a glass filter, grade 3) was added drop by drop until a faint precipitate appeared (about 5 ml. were required), after which the precipitate was just redissolved by the addition of a few drops of water. The solution was then cooled to 0°, and left overnight to crystallize in a stoppered bottle, which it almost filled to leave a minimum of air-liquid surface. The crystals, V, were centrifuged at 0°. Recrystallization was carried out by dissolving the crystals in $(\text{NH}_4)_2\text{SO}_4$ solution, as for precipitate IV, and repeating the operation above.

Analytical methods. Protein N was determined by the method of Falconer & Taylor (1946). A conversion factor of 6.25 was adopted arbitrarily to calculate protein.

The action of *Q*-enzyme on amylose or starch can be followed by sampling and staining with iodine solution. The stain becomes progressively less blue as the reaction proceeds (Bourne, Macey & Peat, 1945), and finally takes on the hue characteristic of amylopectin. This colour change can be used for the quantitative estimation of *Q*-enzyme and for a determination of activity/unit weight by applying an empirical relation described earlier (Gilbert & Swallow, 1949) for the rate of decrease of optical density at 6800 Å. It was found then that if a non-retrograding 'soluble' starch is used as substrate, the optical density *D* falls approximately exponentially to its final value D_∞ , thus simulating a first-order reaction. The apparent velocity constant of the reaction is proportional to the enzyme concentration under the usual conditions of testing, and therefore the activity of the enzyme may be expressed by velocity constant/enzyme concentration. D_∞ is an empirical constant which varies slightly with each preparation of starch, and is only approximately equal to the optical density of the products of reaction.

Q-enzyme activity was determined at 20°, using a test solution prepared from constituents in the proportion: 2 vol. enzyme solution, 1 vol. starch solution (10 mg. 'Analar' soluble starch/ml. of water), 2 vol. citrate buffer solution (0.2 M-disodium hydrogen citrate, NaOH such that the pH of the test solution was 7.0). 0.5 ml. samples were removed at intervals and stained with I_2 (1 mg.)-KI (10 mg.) in a solution of final volume 50 ml. The optical density of the samples was measured in 4 cm. cells with a Hilger-Spektrabsorptiometer (6800 Å., Ilford Spectrum

Filter no. 608). $\log (D - D_\infty)$ was plotted against time, *t* (min.), and activities calculated from the slope of the initial straight part of the line using the relation

$$\text{Activity} = - \frac{2.30 \, d \log (D - D_\infty)}{c \, dt},$$

where *c* is the concentration of the enzyme in the test-solution in mg./ml. The units of activity are therefore $\text{min.}^{-1} \text{mg.}^{-1} \text{ml.}$ If the concentration of the enzyme was not known or was immaterial, the activity of the solution was expressed as $-2.30 \, d \log (D - D_\infty)/dt \, \text{min.}^{-1}$.

Velocities of reaction at 10, 20 and 30° were compared using samples of solution of the crystallized enzyme prepared in 0.2 M-citrate buffer pH 7.0. The tests were carried out simultaneously and curves *A*, *B* and *C* in Figs. 3 and 4 obtained at these respective temperatures. Meanwhile, since *Q*-enzyme is thermolabile (Barker, Bourne & Peat, 1949), a measure of the rate of decay of the enzyme at 30° was obtained by keeping two samples of the enzyme solution at 30° for 88 and 176 min. respectively before adding starch. The velocities of reaction were then measured at 30° to give curves *D* and *E* in Figs. 3 and 4 (p. 184). Control solutions were kept for similar times at 10°.

Reducing power was determined by the method described by Barker *et al.* (1949). The values listed in Table 1 may be compared with one another since, under the standard conditions of testing, the volume of the test solution in each case corresponded to 1.12 times the volume of the potato juice from which the added enzyme was derived.

Phosphorylase was determined under approximately the conditions found by Green & Stumpf (1942) to lead to proportionality between enzyme concentration and phosphate liberation. A solution consisting of 'Analar' soluble starch (0.5 ml. 5% solution), 0.5 M-citrate-NaOH buffer, pH 6.0 (0.5 ml.) and enzyme solution (1.5 ml.) was brought to 35.5°, and 0.1 M-dipotassium glucose-1-phosphate (1 ml.) then added. After 12 min. 6% trichloroacetic acid (5 ml.) was added to the test solution, the precipitated protein centrifuged down, and the supernatant analysed for inorganic phosphate by the method of Allen (1940). Inorganic phosphate not due to the action of the enzyme was allowed for by running a control solution at the same time in which the enzyme had been inactivated by trichloroacetic acid before the addition of glucose-1-phosphate.

RESULTS

Electrophoretic analysis of Q-enzyme prepared by precipitation with lead acetate and ammonium sulphate. A sample of enzyme prepared by the method described by Barker *et al.* (1949) (their fraction '*Q*₁') was dialysed for 48 hr. at 0° against 0.01 M-ammonium sulphate, which had been brought to an ionic strength of 0.1 with sodium chloride, and to a pH of 8.2 with ammonia. Analysis in a large sectional cell by the electrophoretic method of Tiselius showed that the constituent proteins were negatively charged and that at least six components were present (Fig. 1*a*). By testing small samples of solution removed from the ascending and descending boundaries, it was further shown that *Q*-enzyme activity was associated only with the protein moving with the highest mobility. This was a very small

fraction of the whole, and from a number of such experiments it was concluded that at the most only 5% of the protein in the preparation was *Q*-enzyme. The very low mobility of the bulk of the protein impurity suggested that a considerable purification could be effected by electrophoresis. This was attempted, but was not pursued when it was found that the enzyme was too unstable in solution to survive the long procedure required.

Using the above figure for the activity of the crystallized enzyme (V), the concentration of *Q*-enzyme in the original potato juice was calculated to be approximately 16 mg./l.

Examination of precipitate III. Before subjecting precipitate III from the ethanol-fractionation process to further fractionation in ammonium sulphate solution, it was examined by electrophoresis, and by the solubility test of Falconer &

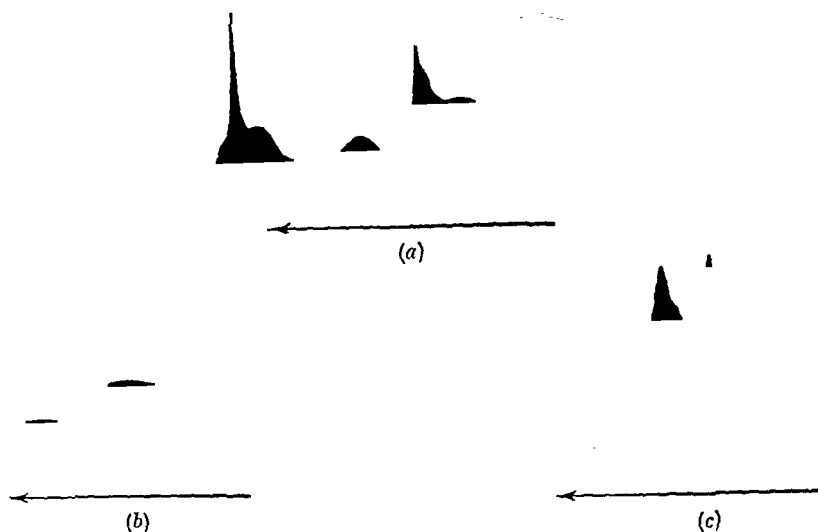


Fig. 1. (a) Electrophoresis of *Q*-enzyme prepared by precipitation with lead acetate and ammonium sulphate. Ascending boundary after 124 min. (b) Electrophoresis of precipitate III. Ascending boundary after 170 min. (c) Electrophoresis of precipitate IV. Ascending boundary after 76 min.

Ethanol and ammonium sulphate precipitation and crystallization of Q-enzyme. The enzyme activity/mg. protein/ml., and the yield of enzyme at different stages of the preparation of crystalline *Q*-enzyme are given in Table 1. Precipitates are numbered as under Methods. The yield is shown relative to precipitate I, since a direct determination of the *Q*-enzyme content of untreated juice is not possible because of the presence of starch precipitants and amylases. The figures are averages of the results of several experiments, each carried out with 100 ml. of potato juice. The relative yield has usually been less if considerably more juice were taken.

Taylor (1946). Philpot schlieren photographs (Fig. 1b), taken after electrophoresis in 0.1M-citrate-NaOH solution, pH 7.5, revealed a component of very low mobility representing about 60% of the protein of precipitate III, a component of intermediate mobility (25%), and a relatively fast component (15%). *Q*-enzyme activity was shown to be associated only with the fast component. Precipitate III did not lose any appreciable amount of activity during either electrophoresis or the preliminary dialysis at 0° for 24 hr.

The results of the solubility-activity test carried out on precipitate III at 0° and pH 6.2 in ammo-

Table 1. Activity and yield at stages in the preparation of crystalline *Q*-enzyme

Ppt.	Activity at 20° min. ⁻¹ mg. ⁻¹ ml.	Relative yield	Reducing power of test solution after 20 hr. as apparent conversion to maltose (%)	Phosphorylase activity relative to potato juice (=1.0)
I	0.04	1.0	12.5	0.05
III	0.15	0.9	8.6	0.012
IV	0.40	0.7	4.7	<0.007
V	2.4	0.7	1.9	<0.0016

nium sulphate solutions of increasing ionic strength are shown in Fig. 2. Protein began to precipitate at an ionic strength of 3.0 from a solution of 0.18 mg. N/ml. (Kjeldahl). Simultaneously, the activity of the residual solution began to fall, indicating that *Q*-enzyme was present in the least soluble component. According to Fig. 2, section *AB*, the protein precipitated had a constant activity/unit weight until about 80% of the activity had been precipitated (ionic strength 3.6). A short extrapolation of *AB* to zero activity of the solution showed that if the precipitated component could be treated as homogeneous the enzyme activity was associated with a component representing 34% of the total protein.

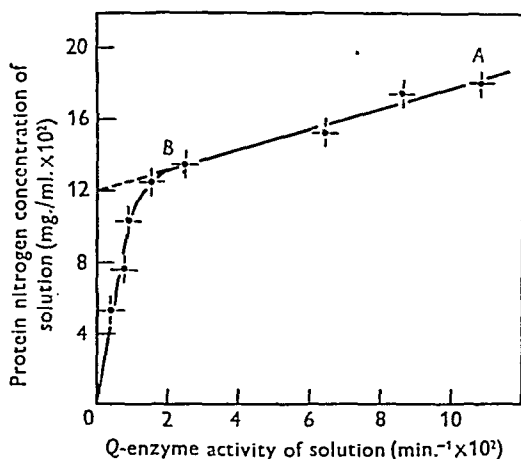


Fig. 2. Solubility-activity curve for precipitate III.

However, in spite of the linearity of *AB*, this component evidently was not a pure protein since the electrophoretic data of Fig. 1*b* had led to a figure of 15% as a maximum. In conformity with this, electrophoretic analysis (pH 7.5, 0.1M-citrate-NaOH solution) of the protein precipitated at ionic strength 3.6 (Fig. 1*c*) showed the presence of two components which corresponded in mobility to the two faster components of Fig. 1*b*. There was no sign in Fig. 1*c* of the slow component in Fig. 1*b*, which therefore must remain in solution at ionic strength 3.6.

Stability of *Q*-enzyme. The crystallized enzyme does not lose more than 5% of its activity during freeze-drying from 0.05M-citrate-NaOH buffer pH 7.0 (cf. Barker, Bourne, Wilkinson & Peat, 1950) and is then perfectly stable at 0° in the dry state. In solution in 0.2M-citrate-NaOH buffer pH 7.0 the activity decays to half in 11 days at 0° or in 4 days at 17°. It will be seen in the next paragraph that the decay at 30° is extremely rapid.

Effect of temperature on the action of *Q*-enzyme on starch. The relative rates of reaction at 10, 20 and 30° were calculated from the initial slopes of the lines *A*, *B* and *C* respectively in Fig. 4 to be 0.47, 1.0,

1.0. At 30° the activity rapidly decayed during the course of the test, as shown by the progressive decrease in slope of curve *C*, and after 120 min. had fallen to 40% of its initial value. The two samples of enzyme that had been kept at 30° in the absence of

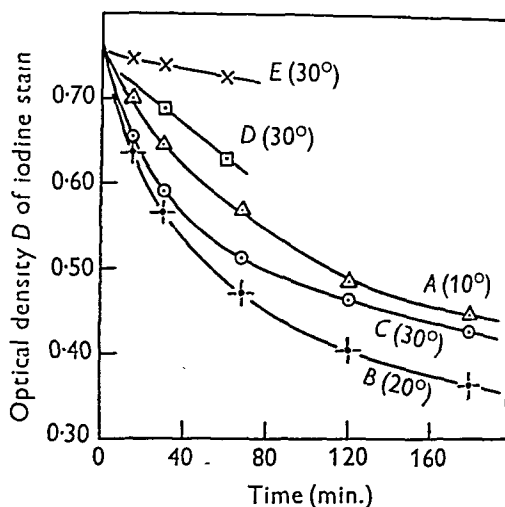


Fig. 3. The effect of temperature on the action of crystallized *Q*-enzyme on starch solutions.

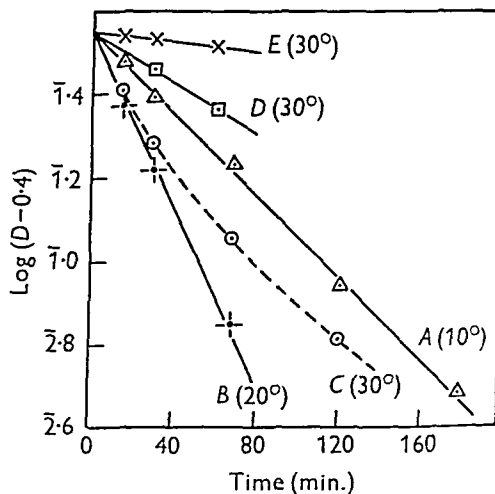


Fig. 4. Activity of *Q*-enzyme. $\log (D - D_{\infty})$ as a function of time.

starch for 88 and 176 min., respectively, before being tested, had decayed even more rapidly (curves *D* and *E* respectively) and retained only 29 and 6% of their activity. The solutions kept at 10° for similar times before the addition of starch showed no loss of activity.

Fresh starch was added to the test solution corresponding to curve *C* after it had stood for 3 hr. at 30° and had cooled slowly to room temperature for 16 hr. Appreciable activity was still present, since the optical density of the iodine stain fell from 0.84 to 0.61 in 120 min. at 20°, and to 0.42 in 270 min.

Activation of Q-enzyme by ions. A suspension of crystals of the enzyme in ammonium sulphate solution was dissolved in water. The ionic strength of the resulting solution was estimated to be 0.017 by passing a sample through an exchange resin and estimating the hydrogen ion produced. The solution was diluted to an ionic strength of 0.010, and then, as a precaution against suspended enzyme which might later be dissolved at higher salt concentrations, filtered through a Whatman no. 42 paper and a G-4 sintered-glass funnel under slight positive pressure. Samples were then added to equal volumes of solutions of starch at pH 7.0 and various ionic strengths equal to or greater than 0.01 (precipitation of the enzyme by reduction in salt concentration thereby being guarded against), and the corresponding activities determined. These are given in Table 2. Further, to test that the effects were not due to suspended enzyme, a portion of the enzyme solution of ionic strength 0.01 was diluted with an equal volume of ammonium sulphate solution of ionic strength 0.01 and the resulting solution tested separately for activation, the results being indicated by the starred entries in the table.

Table 2. *Activation of Q-enzyme by ions. Activity measured in solutions of increasing ionic strength*

Ionic strength of solution	Activity $\times 10^2$ (min. ⁻¹)
0.01	1.7
0.05	2.9
0.25	4.3
1.25	6.5
0.01*	1.0
1.25*	3.0

* Enzyme solution diluted with $(\text{NH}_4)_2\text{SO}_4$ solution before testing. See text.

Dependence of the yield of enzyme on the season and on the variety of potato. Difficulty in the preparation of Q-enzyme during a certain period of the year was noted by Barker *et al.* (1949). The same trouble was also encountered in the present investigation. In the middle of March, King Edward potatoes harvested in the previous autumn were found to be almost devoid of active enzyme, although 3 weeks previously their activity had been normal. This change coincided with the beginning of sprouting in the potatoes, and at the same time activity due to an amylase of lower solubility than Q-enzyme disappeared. The amylose precipitant also increased considerably and the precipitates became oily and more difficult to work with. A standard method of fractionation could not be devised until fully grown potatoes were available in August.

With the exception of 'Ninetyfold' potatoes, all the varieties of white-skinned potatoes tested gave

very low yields of Q-enzyme in comparison with red-skinned varieties, among which there was little to choose.

DISCUSSION

The original method for preparing Q-enzyme (Bourne & Peat, 1945), after modification by Barker *et al.* (1949), consists of precipitating potato juice with lead acetate, dissolving the precipitated protein and precipitating the enzyme with ammonium sulphate. Although the method has served for the characterization of the enzyme, it is evident from the electrophoresis results above that it does not differentiate sufficiently between the enzyme and the numerous proteins which occur in potato juice. Much greater selectivity is attained by precipitation at low ionic strength with ethanol (Cohn *et al.* 1946, 1950). The number of variables available in this method make it very suitable for problems such as the present one where a minor constituent of a mixture of proteins has to be isolated.

It has to be remembered, however, that ethanol quickly inactivates Q-enzyme if certain limits of temperature and concentration are exceeded (compare the inactivation by ethanol reported by Bourne *et al.* 1945). Even under the conditions described above for the preparation, the enzyme should not be left too long in contact with ethanol, e.g. overnight. Ammonium sulphate solution is therefore a better medium than ethanol solution from which to attempt crystallization.

The influence of the season and of the variety of potato on enzyme content makes it advisable to carry out preliminary experiments before attempting a large-scale extraction of a new batch of potatoes. It is then possible to decide whether sufficient enzyme is present to be worth extraction, and whether any slight alterations in the concentrations of ethanol specified above need be made to achieve optimum results with the particular batch.

The enzymic properties of the crystallized enzyme resemble those described for the amorphous form in the references cited. The ability of the enzyme to form reducing groups when incubated with amylose is somewhat decreased but still remains, although detectable by reducing power measurements only after long incubation. It is confirmed that Q-enzyme is activated by ammonium sulphate as described by Gilbert & Swallow (1949). The experiments were conducted with particular care to eliminate any possibility that enzyme suspended, but not dissolved, at low ionic strengths was passing into solution at higher ionic strengths and simulating activation.

The lability of the enzyme is demonstrated by the results obtained for the reaction with starch at 30°

(curve C, Fig. 4). It is obvious that for valid comparisons of reaction rate at different temperatures the initial slope of the reaction curve must be obtained. When this is done it is still found that the velocity of reaction reaches a maximum somewhere between 20 and 30° and that an 'optimum' temperature does not result from the onset of inactivation.

The experiments in which Q-enzyme was kept at 30° for some time before adding starch and testing activity confirm this lability. Thus in 2 hr. only 6% of the original activity remained. It is noteworthy that the decay of activity at 30° in the solution which contained starch throughout was much less rapid, a result which suggests that starch or its reaction product tends to stabilize the enzyme.

SUMMARY

1. Potato Q-enzyme has been prepared in crystalline form and with high activity by a method involving precipitation with ethanol at low temperature and low ionic strength.

2. Electrophoretic analysis has been used to follow the course of the purification.

3. Activation of the enzyme by ions has been confirmed, and the effect of temperature on the activity of the enzyme has been examined.

The authors are indebted to Prof. M. Stacey, F.R.S., for his interest in this work, and to the Royal Society and the Dunlop Rubber Co. Ltd., for grants for equipment. One of them (A. D. P.) wishes to record his thanks to the University of Birmingham for the award of an A. E. Hills research scholarship.

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Enzymes of the Potato Concerned in the Synthesis of Starch

2. THE SEPARATION OF PHOSPHORYLASE

By G. A. GILBERT AND A. D. PATRICK

Department of Chemistry, The University, Edgbaston, Birmingham

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Several accounts have been published of attempts to purify the starch phosphorylase of the potato. The original technique of Hanes (1940), in which the enzyme was precipitated from potato juice with

ammonium sulphate, has been modified by, among others, Green & Stumpf (1942), Meyer & de Traz (1944), Weibull & Tiselius (1945), Bourne & Peat (1945) and Barker, Bourne, Wilkinson & Peat (1950).

The degree of purity which can be reached by these methods is probably about 5 %, with respect to protein, according to an electrophoretic analysis carried out by Weibull & Tiselius (1945). The electrophoretic analysis would not, however, have differentiated proteins with the same mobility as the enzyme, but a similar estimate from ultracentrifugation experiments by these authors lent support to the figure. The protein impurity is probably mostly inert, but Barker *et al.* (1950) have shown that their preparation still retains considerable phosphatase activity, and that traces of *Q*-enzyme and amylases may be present. A threefold greater purification is reported by Sumner, Chou & Bever (1950) for the phosphorylase of jack bean after a treatment including precipitation from concentrated acetone solution, but only a low yield is claimed.

As described in Part 1 of this series (Gilbert & Patrick, 1952), *Q*-enzyme can be isolated from potato juice in good yield, and in satisfactory purity, by a method of protein purification developed by Cohn *et al.* (1946) and Cohn *et al.* (1950), based on fractionation by ethanol precipitation. Green & Stumpf (1942) and Meyer & de Traz (1944) concluded that ethanol causes complete deactivation of potato phosphorylase even at 0°, but in contradiction to this conclusion it was found that, under the conditions adopted for the preparation of *Q*-enzyme, phosphorylase was stable in the presence of considerable concentrations of ethanol, and could be prepared from the ethanol solution remaining after the precipitation of *Q*-enzyme from potato juice. This residual solution was therefore used as a starting material for the following experiments.

METHODS

Potato juice (100 ml.) was precipitated successively at 11 and 14.9 % ethanol concentration as described for the preparation of *Q*-enzyme in Part 1. The supernatant was then adjusted at -5° to a concentration of ethanol of 17.7 % by adding 0.087 ml. of 50 % (v/v) ethanol-citrate solution (0.01 M-disodium hydrogen citrate, NaOH to pH 6.0)/ml. of supernatant, with the precautions of slow addition and thorough but gentle stirring previously described. After removal by centrifugation at -5°, the precipitate was dissolved at 0° in 20 ml. of 0.01 M-citrate solution of pH 6.0 and then reprecipitated at -5° and 20 % ethanol concentration by adding 0.67 ml. of the 50 % ethanol-citrate precipitant/ml. The precipitate *A* was dissolved at 0° in 5 ml. of 0.05 M-citrate solution of pH 6.0 and freeze-dried.

The powder (about 100 mg.) was extracted, with gentle but frequent stirring, for 30 min. at -5° with 25 ml. of 20 % ethanol-citrate solution (pH 6.0, 0.01 M). After centrifugation at -5°, the insoluble residue was extracted at -2° with stirring for 30 min. with 50 ml. of 11 % ethanol solution containing 0.01 M-sodium acetate (HOAc to pH 6.0) (this solution representing a considerable decrease in ionic strength in comparison with the previously used citrate solution of the same molarity). After centrifugation at -2°,

the residue, *B*, was extracted for 30 min. with stirring at 0° with 50 ml. of freshly prepared sodium acetate solution (0.01 M, pH 6.0) containing 0.000167 M-zinc acetate. The extracted residue, *C*, was dissolved in 50 ml. of 0.01 M-citrate solution of pH 6.0 and precipitated at 0° from 22 % (w/v) (NH₄)₂SO₄ by the addition of 0.79 ml. of 50 % (w/v) (NH₄)₂SO₄ (pH to 6.0 with NH₃) to each ml. of solution. The precipitate was discarded and the supernatant brought to a concentration of 35 % (w/v) (NH₄)₂SO₄ by the addition of 0.87 ml. of 50 % solution/ml. to give precipitate *D*. This precipitate was dissolved in 5 ml. of 0.05 M-citrate buffer solution of pH 6.0 and freeze-dried.

Modification. A higher recovery of the phosphorylase of the potato juice was obtained when the concentration of ethanol for the initial precipitation of the phosphorylase was raised from 17.7 % ethanol to 21 %, and the precipitate then washed with 30 ml. of 21 % ethanol solution containing 0.01 M-citrate of pH 6.0. The effect on the purity of the final product has not yet been studied.

Measurement of enzyme activity. (1) Phosphorylase activity was measured by the method of Green & Stumpf (1942), after slight modification of their conditions, as described in Part 1. The unit used is the weight in mg. of elementary phosphorus produced/min./ml. of test solution for a concentration of protein in the test solution of 1 mg./ml.

(2) A portion of the same solution of precipitate *D* (in 0.01 M-citrate solution, pH 6.0) that was tested for its phosphorylase activity, was tested for *Q*-enzyme and amylase activity by the method of Barker, Bourne & Peat (1949). The amylose test solution (14 ml.) contained 0.05 mg. protein. Protein N in the solution of *D* was determined by precipitation of the protein with trichloroacetic acid, followed by washing to remove (NH₄)₂SO₄ and Kjeldahl estimation according to Falconer & Taylor (1946). A factor of 6.25 was used to convert weight of nitrogen to weight of protein. Samples of the digest were examined after 2 and 20 hr. Control solutions were incubated (a) with water instead of enzyme solution, (b) with water instead of amylose solution.

(3) Phosphatase activity was estimated using, (a) sodium glycerophosphate or (b) glucose-1-phosphate as substrate.

(a) Enzyme solution (5 ml.) was added to 10 ml. of a buffer solution containing 0.15 M-sodium glycerophosphate and 0.2 M-disodium hydrogen citrate (pH to 6.5 with NaOH), and the solution incubated at 20°. A control solution contained water instead of enzyme solution. Free phosphate in these solutions was estimated periodically by the method of Allen (1940).

(b) Enzyme solution (12 ml.) was added to a solution prepared from 0.1 M-glucose-1-phosphate dissolved in 0.5 M-citrate-NaOH buffer of pH 6.0 (12 ml.) and 0.1 % A.R. soluble starch (4 ml.). The test solution was incubated at 20°. At intervals, 5 ml. samples were withdrawn and tested for liberated glucose by the method of Shaffer & Hartmann (1921). A known weight of glucose (about 0.2 mg.) was added to each 5 ml. sample before this test.

RESULTS

The absolute activities of the residues and precipitates, lettered as under Methods, obtained during the course of the fractionation are given in Table 1.

Table 1. Activities of phosphorylase preparations

Preparation	Activity (mg. P/min./ml./ mg. protein/ml.)	Yield relative to ppt. A
A	0.15	1
B	0.29	0.9
C	0.61	0.7
D	0.98	0.6



Fig. 1. Electrophoresis of potato phosphorylase after precipitation from potato juice with ethanol (precipitate A). An arrow indicates the centre of the phosphorylase boundary.

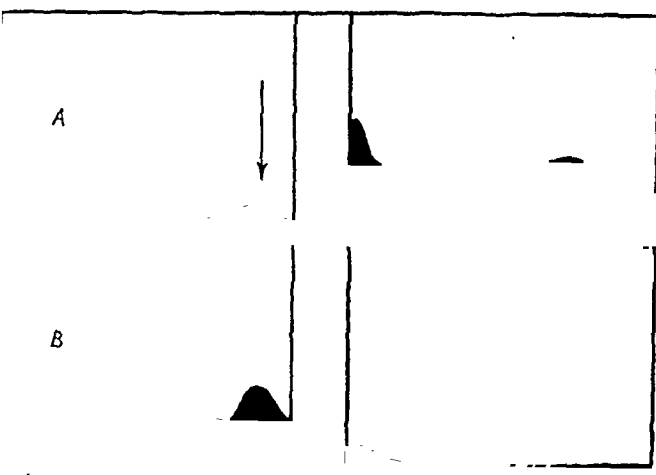


Fig. 2. Philpot-schlieren photograph of sectional electrophoresis U-tube, illustrating the separation of the component (arrow) of precipitate A with phosphorylase activity (ascending boundary A), and of the components without activity (descending boundary B).

Preparation A contains about 70 % of the phosphorylase originally in the potato juice, and about 20–30 % of the original *Q*-enzyme. It can be seen that precipitate D is about seven times more active with respect to unit weight of protein than preparation A.

In the test for amylases carried out on precipitate D, no increase in reducing power of the amylose test solution was detected even after incubation for 20 hr. The optical density (6800 Å.) of the amylose solution fell from 1.08 to 1.04 in 20 hr. and in the control solution from 1.05 to 1.02. There was thus no indication in these tests of the presence of either

amylases or *Q*-enzyme in the phosphorylase preparation.

Electrophoresis of precipitate A. Electrophoretic analysis of precipitate A was carried out in 0.1M-sodium citrate-NaOH buffer at pH 7.0 after dialysis at 0° for 18 hr. The protein migrated towards the anode and was resolved into at least four components during 138 min. (Fig. 1). The fastest of these components, which has been indicated by an arrow in the figure, represented about 6 % of the total protein, the slowest, which hardly moved, about 60 %. By adjusting the positions of the boundaries and sliding the sections of the Tiselius cell, a portion of the fast component was isolated in an electrophoretically pure state from the ascending boundary (Fig. 2). When tested for phosphorylase activity this isolated fraction was found to liberate 0.11 mg. phosphorus/ml. of test solution in 12 min. compared with 0.0024 mg. liberated by the corresponding fraction isolated from the descending boundary which had been freed only from this component. The fast component therefore contained the whole of the phosphorylase.

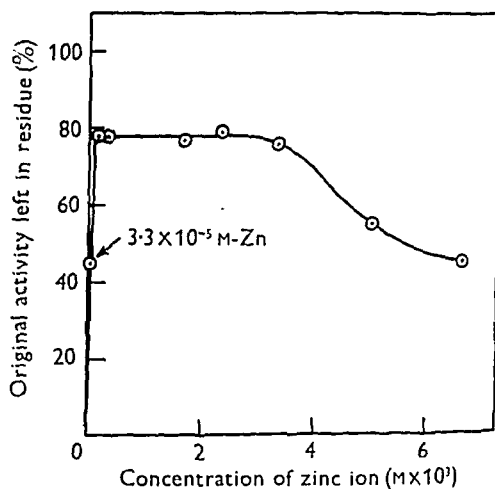


Fig. 3. The residual phosphorylase activity of residue B after extraction with dilute solutions of zinc acetate at pH 6.0.

The effect of extraction with zinc acetate solution on the phosphorylase activity of residue B. In the course of determining whether zinc salts could be used to effect any purification of phosphorylase preparations, residue B was extracted with 0.01M-sodium acetate solution, pH 6.0 (50 ml.), containing various quantities of zinc acetate. Determination was then made of the protein content and the activity of the extracted residues and of the solutions. It was discovered that if the concentration of zinc ion was above about $3.3 \times 10^{-5} M$ a considerable loss of activity occurred from the residue, but that between this concentration and $1.7 \times 10^{-4} M$ -zinc a constant decrease of about 20 % took place as illustrated in

Fig. 3. After the extraction procedure, only the residues were active and no activity was detected in any of the zinc solutions.

In order to throw light on these results, the activity of residue *B* from the stage before the treatment with zinc was measured in a normal test solution and also in a test solution containing 0.02M-zinc acetate. The rate of liberation of phosphorus proved to be the same, showing that the 20% loss in activity was not due to any traces of zinc retained in the precipitate during the treatment. (The citrate in the test solution prevented precipitation of the protein by the zinc.)

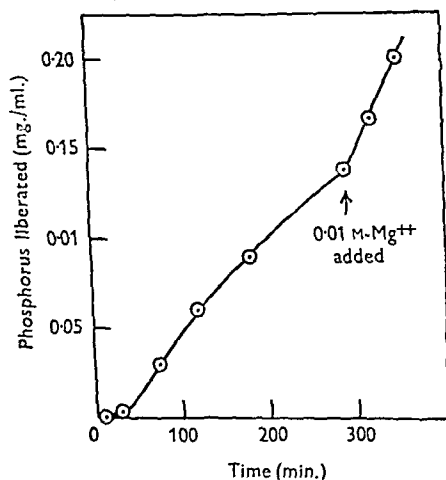


Fig. 4. The hydrolysis of sodium glycerophosphate by partially purified potato phosphorylase (precipitate *A*).

In spite of the loss in total activity incurred, it was decided to include the zinc treatment in the fractionation scheme since there was a net increase in activity/mg. protein (Table 1).

Tests for phosphatase. No phosphatase activity was detected in any of the precipitates or residues when these were incubated with glucose-1-phosphate, and only a normal synthesis of amylose occurred, without a measurable release of reducing sugar. However, when incubated with glycerophosphate, precipitate *A* was found to have considerable phosphatase activity. A solution of the precipitate, concentrated 2.5 times with respect to the potato juice from which it was derived, caused a 4.4% hydrolysis of 0.1M-sodium glycerophosphate to free phosphate in 290 min. This rate of hydrolysis was doubled by the addition of magnesium sulphate to 0.01M. An induction period occurred at the beginning of the reaction (Fig. 4).

Extraction with 11% ethanol (to give residue *B*) removed almost all the phosphatase, only 0.2% hydrolysis of 0.1M-sodium glycerophosphate then occurring in 240 min. The last trace of phosphatase

activity was removed (none being detected even with 0.01M-Mg⁺⁺ in the test solution) during extraction with 0.000167M-zinc acetate solution in the preparation of residue *C*.

Freeze-drying of precipitate *D*. At least 92% of the phosphorylase activity of this precipitate was retained during freeze-drying from solution in 0.05M-citrate buffer solution of pH 6.0. These are the conditions recommended by Barker *et al.* (1950) except for the use of a lower salt concentration.

DISCUSSION

Fractional precipitation and extraction of potato juice with ethanol, zinc acetate and ammonium sulphate has led to the preparation in reasonable yield of very active phosphorylase. This would seem to have about nine times the activity per unit weight of the preparations discussed by Weibull & Tiselius (1945). Even so, the electrophoretic analysis of precipitate *A*, and the electrophoretic and ultracentrifugation experiments of Weibull & Tiselius on their preparation, suggest that an increase in purity of at least twice remains to be achieved.

The decrease of one-fifth in the activity of phosphorylase during extraction with very dilute solutions of zinc acetate is not yet understood. It could arise from the presence of two rather similar enzymes, only one of which is inactivated by zinc, or from the displacement of an essential cation by zinc, but it cannot be due simply to the retention of traces of zinc by the extracted residue.

Amylase activity was not detected in the purified phosphorylase. The test described, however, cannot be regarded as a satisfactory criterion of purity for phosphorylase required for studies involving amylose of high molecular weight, and it becomes desirable to use measurements of molecular weight directly to prove complete freedom from amylase.

SUMMARY

1. Potato phosphorylase has been prepared by a method involving precipitation from potato juice with ethanol followed by extraction with zinc acetate and precipitation with ammonium sulphate.
2. The phosphorylase preparation has an activity about nine times greater than that of preparations previously described.
3. No phosphatase, amylase or *Q*-enzyme activity has been detected in the product.

The authors are indebted to Prof. M. Stacey, F.R.S., for his interest in this work, and to the Royal Society and the Dunlop Rubber Co. Ltd., for grants for equipment. One of them (A. D. P.) wishes to record his thanks to the University of Birmingham for the award of an A. E. Hills research scholarship.

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The Component Acids of the Milk Fat of a Grey Atlantic Seal

By M. L. MEARA

Department of Industrial Chemistry, University of Liverpool

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The milk fats of many land animals are characterized by the presence of fatty acids of lower molecular weight than those obtaining in their respective depot fats. This phenomenon is most marked in the case of the milk fats of the ruminants which contain appreciable amounts, especially when expressed as a molar percentage, of the lower even-membered saturated acids from butyric to myristic, which are either absent or occur only in small amounts in their respective depot fats. It is not so pronounced, however, in the case of the milk fats of other herbivora, e.g. horse and ass, while sow milk fat has been shown (De la Mare & Shorland, 1944) to resemble closely that of a normal pig depot fat. The Reichert and Polenske values recorded for milk fats of a number of other animals (e.g. cat, dog and mouse) also indicate the absence therefrom of short-chain acids.

Little, however, is known of the composition of the milk fats of marine animals. Klem (1935) has recorded data for various species of whales, and indicated that the fat content of the milk ranged from 19 to 46 %, thus far exceeding that found in the milk of land animals (usually 3–6 %). He also noted that the mean unsaturation of the milk fat of a blue whale was considerably higher (iodine value 171.8) than that of the fat from any other site in the same animal (iodine values 94.0–102.8), though these latter values are considerably lower than the values obtained for fat obtained from the same sites in healthy males and non-lactating females of the same

species (iodine values 120–140). He further showed that the blue whale milk fat contained the following acids: myristic 8.4, palmitic 16.8, stearic 1.8, unsaturated acids C_{14} 1.2 (–2.0H), C_{16} 6.2 (–2.0H), C_{18} 26.8 (–3.3H), C_{20} 25.9 (–8.5H), C_{22} 12.9 (–11.0H) % (w/w). Similarly, Schmidt-Nielsen & Frog (1933) showed that there are no acids of lower molecular weight than lauric acid present in the milk fat of the finner whale.

An opportunity for further study of the milk fat of a marine animal was made possible by the kindness of Prof. E. C. Amoroso, Dr S. J. Folley, F.R.S., and Dr S. J. Rowland, who placed a small quantity of the milk of a grey (Atlantic) seal (*Halichoerus grypus*), caught off the Pembrokeshire coast in 1950, at the disposal of this laboratory.

METHODS AND RESULTS

The milk was pale cream in colour, of a thick viscous consistency and had a pronounced fishy odour. On extraction of 235 g. milk with light petroleum (b.p. 40–60°) (emulsions readily broken by addition of ethanol) 118 g. (50.0 %, w/w) of a pale oil, saponification equivalent 295.9, iodine value 146.2, unsaponifiable matter 1.5 %, was obtained. The fat (101 g.) gave 95.2 g. mixed fatty acids, after saponification and acidification of the soaps. The steam distillate from the mixed fatty acids required 1.8 ml. 0.1 N-KOH to neutralize the steam volatile acids, this being equivalent to 0.03 g. decanoic acid (or a proportionally smaller amount of the lower steam-volatile acids) indicating the virtual absence of this group of fatty acids.

Component fatty acids of the seal milk fat

The mixed acids (86.3 g.) were crystallized first from acetone at -70° , the deposited acids being subjected to a further recrystallization from acetone at this temperature. The deposited acids were then crystallized from ether at -40° . In this manner the acids were resolved into three fractions consisting of mainly saturated, mainly mono-ethenoid and mainly polyethenoid acids respectively, their proportions and unsaturation being recorded in Table 1.

Table 1. *Low temperature crystallization of seal milk fatty acids*

	Weight		Iodine value
	(g.)	% of total	
A Insoluble in ether at -40°	16.85	19.5	3.4
B Soluble in ether at -40°	22.30	25.8	87.5
C Soluble in acetone at -70°	47.15	54.7	234.2

After methylation, according to the procedure recommended by Bjarnason & Meara (1944), each group of esters was fractionally distilled, the analytical data obtained for each ester fraction being recorded in Table 2, together with the spectroscopic analysis of the acids recovered from fractions B4 and C3. From these results the composition of each ester fraction is calculated according to the methods adequately described by Hilditch (1947, pp. 498-510) and Gupta & Hilditch (1951), and therefrom the composition of each group of acids, leading subsequently to the component acids of the whole fat (Table 3).

DISCUSSION

It is now established that whilst the component fatty acids of whale oils vary but little from season to season (Armstrong & Allan, 1924; Tveraaen, 1935; Hilditch & Maddison, 1948), seal blubber oils show an abnormally large variation in the proportions of their component fatty acids (Hilditch & Pathak, 1949; Winter & Nunn, 1950*a, b, c*). These latter authors have shown (1950*b*) that significant differences occur, not only in the blubber oils of the elephant seals from different localities (Heard Island and Macquarie Island), but also in the blubber oils of elephant seals caught in the same locality (Heard Island) and concluded that the influence of any of the three factors, diet, sex and species, was not sufficient to account for the observed differences.

Comparison of the component acids recorded in Table 4 shows that no fundamental difference exists between the blubber oils of the seals so far reported and the seal milk fat, such as occurs between the depot and milk fats respectively of the ruminants and some other land animal species.

There are, however, notable differences in the proportions of the component acids of the seal milk fat and the blubber fat of an animal of the same species, and indeed differences from the blubber fats of all the species of seals so far recorded. Thus,

although the content of both myristic and stearic acids in the milk fat is of the same order as that which obtains in the blubber oils of both British coastal and Antarctic seals, the milk fat differs markedly, in that the palmitic acid content rises to about 16% as compared with the more usual 10-12% in the blubber oils. This increase in the palmitic, and therefore in the total saturated acid content of the milk fat does not appear to be at the expense of any particular unsaturated acid group, if the recorded analysis of grey seal blubber fat can be considered to be typical for that species.

Table 2. *Fractionation data for seal milk ester fractions*

Fraction	Weight (g.)	Saponification equivalent	Iodine value
Methyl esters of acids A			
A1	1.40	245.8	1.1
A2	1.87	266.8	0.8
A3	2.55	269.1	1.0
A4	2.60	269.3	0.8
A5	3.00	270.5	0.5
A6	3.40	278.0	5.6
A7	2.22	333.1*	16.4
Total	17.04	—	—
Methyl esters of acids B			
B1	1.77	262.0	52.8
B2	2.05	286.0	78.2
B3	2.06	294.8	82.3
B4	2.70	295.3	83.7
B5	2.59	295.3	83.9
B6	2.80	297.1	85.5
B7	3.19	297.3	85.8
B8	2.77	298.7	86.1
B9	2.72	323.2*	96.9
Total	22.65	—	—
Methyl esters of acids C			
C1	1.67	241.6	64.2
C2	2.31	263.0	89.4
C3	2.80	267.5	98.0
C4	3.61	271.0	98.2
C5	4.42	281.0	106.7
C6	6.51	292.3	111.6
C7	7.01	302.2	143.8
C8	6.83	325.8	337.6
C9	3.57	334.9	389.6
C10	3.38	336.9	386.9
C11	2.30	338.2	376.4
C12	3.47	363.8*	233.3
Total	47.88	—	—

* Equivalents of esters (freed from unsaponifiable matter): A7, 300.4; B9, 306.7; C12, 330.4.

Spectroscopic analysis of acids recovered from fractions B4 and C3

	B4	C3
$E_{1\text{cm.}}^{1\%}$, 232 m μ . (unisomerized)	—	5
$E_{1\text{cm.}}^{1\%}$, 270 m μ . (unisomerized)	—	1*
$E_{1\text{cm.}}^{1\%}$, 234 m μ . (180°/60 min.)	3*	42
$E_{1\text{cm.}}^{1\%}$, 268 m μ . (170°/15 min.)	1*	17

* Neglected.

Table 3. *Component acids in groups A, B, C, and in the whole seal milk fat*

Acid	A (19.5%)* %†	B (25.8%)* %†	C (54.7%)* %†	Total fatty acids excluding unsaponifiable	
				(%, w/w)	(% by mol.)
Myristic	8.7	0.9	1.5	2.8	3.4
Palmitic	71.0	4.5	2.2	16.4	17.8
Stearic	14.4	—	—	2.8	2.8
Unsatd. C ₁₄	0.1 (-2.0)	0.8 (-2.0)	2.6 (-2.0)	1.7 (-2.0)	2.0
Unsatd. C ₁₆	0.5 (-2.0)	5.3 (-2.0)	20.3 (-2.2)	12.7 (-2.2)	13.9
Unsatd. C ₁₈	2.5 (-2.0)	80.3 (-2.0)	27.8 (-2.7)	36.6 (-2.3)	36.3
Unsatd. C ₂₀	1.4 (-2.0)	7.6 (-3.7)	20.5 (-7.2)	13.6 (-6.6)	12.4
Unsatd. C ₂₂	—	—	24.4 (-11.2)	13.4 (-11.2)	11.4
Unsaponifiable	1.4	0.6	0.7	—	—

* Group as % (w/w) of total fat.

† Component acids as % (w/w) of group.

Figures in brackets denote mean unsaturated of each group of acids (Hilditch, 1947, p. 23).

Table 4. *Component fatty acids of seal blubber oils and grey seal milk fat (%, w/w)*

	Elephant seal			Leopard seal,	Common seal,		Grey Atlantic seal,	
	Macquarie Island		Heard Island	Heard Island	Norfolk coast		Pembroke coast	
	Mature male	Mature female	mature male	mature male	Suckling female	Yearling male	Blubber fat	Milk fat
Iodine value of oil	136.4	153.5	114.8	130.7	140.4	145.4	162.2	146.2
Acid:								
Lauric	0.3	0.1	—	—	—	—	—	—
Myristic	5.2	4.4	3.4	4.0	2.4	2.2	3.7	2.8
Palmitic	11.4	11.5	9.6	7.4	10.3	10.6	10.5	16.4
Stearic	2.4	2.4	2.5	1.6	2.5	4.4	2.0	2.8
Arachidic	—	—	0.3	0.2	0.3	0.3	—	—
Unsatd. C ₁₄	1.7	1.3	1.2	2.1	2.9	2.2	1.6	1.7
Unsatd. C ₁₆	14.5	13.0	10.5	12.7	25.7	20.8	15.5	12.7
Unsatd. C ₁₈	35.1	33.0	39.8	42.3	32.6	33.7	30.8	36.6
Unsatd. C ₂₀	18.8	20.6	18.0	17.3	12.1	13.6	16.5	13.6
Unsatd. C ₂₂	10.6	13.7	14.7	12.4	11.2	12.2	18.1	13.4
Unsatd. C ₂₄	—	—	—	—	—	—	1.3	—
Mean unsaturation of:								
Unsatd. C ₁₄	-2.0	-2.0	-2.0	-2.0	-2.0	-2.0	-2.0	-2.0
Unsatd. C ₁₆	-2.1	-2.1	-2.1	-2.1	-2.1	-2.2	-2.2	-2.2
Unsatd. C ₁₈	-2.7	-2.4	-2.2	-2.2	-2.7	-2.4	-2.7	-2.3
Unsatd. C ₂₀	-5.4	-5.9	-4.0	-4.6	-5.9	-7.2	-5.7	-6.6
Unsatd. C ₂₂	-10.0	-10.8	-7.4	-9.4	-11.0	-11.0	-10.6	-11.2
Unsatd. C ₂₄	—	—	—	—	—	—	-11.0	—

Although there is considerable variation in the amounts of the unsaturated acids of the various blubber oils, the grey seal milk fat does not conform to any type so far examined. Thus the C₁₆ unsaturated acids more nearly resemble those of the blubber fats of the Antarctic seals rather than those from British coastal waters, whereas curiously, with the exception of leopard seal blubber, the C₁₈ unsaturated acid content of the milk fat shows the least resemblance to that of the grey seal blubber fat. Again, although there is no correlation between the C₂₀ unsaturated acids with those of the Antarctic species, the milk fat much more nearly resembles those values recorded for the blubber of the common seal than that of the grey seal, this latter observation also holding true for the C₂₀ unsaturated acids.

It appears, therefore, that although they are broadly similar there may be no simple correlation between the milk fat of the grey seal and that of its blubber fat, neither does any correlation exist with the blubber fat of other species so far examined.

A further point of interest lies in the fact that the grey seal milk fat is considerably less unsaturated (iodine value 146.2) than that recorded for grey seal blubber oil (iodine value 162.2). In this respect seals might seem to differ entirely from whales, since the milk fat of a blue whale was found to be more unsaturated than that of the fat from any other site in the body of the same animal.

Clearly much more information is necessary before the apparent abnormalities of seal oils are understood, and to that end it is hoped that in the

near future an opportunity may be afforded of studying the blubber and milk fats of a seal at various stages during lactation, and also the blubber fat of the calf.

SUMMARY

1. Grey (Atlantic) seal (*Halichoerus grypus*) milk resembles whale milk in that its fat content is of the order of ten times that found in the milks of land animals.

2. The composition of seal milk fat has been investigated: it contains only those acids which occur in its blubber fat.

3. Qualitatively grey seal milk fat resembles that of the blubber oil of the same and of a number of other species, notable differences, however, occurring in the proportions of palmitic and unsaturated acids.

4. Grey seal milk fat is relatively more saturated than that of the blubber oil in contradistinction to that of the blue whale, for which the reverse holds true.

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The Liberation of Chloride Ions from Organic Chloro Compounds by Tissue Extracts

By H. G. BRAY, W. V. THORPE AND D. K. VALLANCE
Department of Physiology, Medical School, University of Birmingham

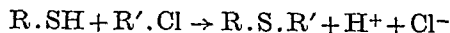
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There have been several reports of the liberation of chloride ions from organic chloro compounds. Liebreich (1869), who introduced chloral hydrate as a hypnotic, stated that this compound was in part excreted as inorganic chloride. This was disputed by von Mering & Musculus (1875), von Mering (1882) and Külz (1882) who found that chloral hydrate was eliminated by man and dogs as urochloralic acid (trichloroethyl- β -glucuronide). Kast (1887) failed to find an increased chloride excretion after administration of chloral hydrate to dogs, although he observed that chloride excretion was considerably increased after chloroform anaesthesia. The increase in man was slight. Willstätter, Straub & Hauptmann (1922) detected only small amounts of urochloralic acid in urine when therapeutic doses of chloral hydrate were given to man or rabbits. They maintained that urochloralic acid was formed when large doses were given, but that chloral was broken down

mainly to inorganic constituents when therapeutic doses were given. The only metabolic product detected in urine after giving trichloroethane to man, rabbits or dogs was inorganic chloride. Akamatsu & Wasmuth (1923), using rabbits, showed that even after large doses of chloral hydrate no more than half could be accounted for as urochloralic acid. More recently Heppel & Porterfield (1948) claimed the discovery of an enzyme in rat liver, kidney and spleen which liberated halide ions from halogenated aliphatic hydrocarbons.

Since metabolic studies on a number of chloro-compounds were being undertaken in this laboratory it was desirable to confirm the existence of an enzyme such as that claimed by Heppel & Porterfield, especially as these workers did not appear to have considered the possibility of chloride ions being liberated as a result of interaction between chloro compounds and SH compounds. Several instances

of reaction between halogen compounds and SH compounds according to the general scheme



have been reported, e.g. halogenated lachrymators (Dixon, 1950), halogenated acetic acids (Dickens, 1933; Rapkine, 1933; Quastel, 1933; Michaelis & Schubert, 1934), lewisite and 2:3-dimercaptopropanol (Thompson, 1950), formation of benzyl mercapturic acid from benzyl chloride in the rabbit (Stekol, 1939). The work here reported describes a preliminary investigation of the liberation of chloride ions from 29 chloro compounds, and a more detailed study of the effect of SH compounds upon the liberation of chloride ions from three of those compounds.

METHODS

Materials. The chloro compounds were all purchased (British Drug Houses Ltd.). DL-Cysteine hydrochloride and L-cystine (British Drug Houses Ltd. and Light and Co. Ltd.) were used. Glutathione was obtained from Roche Products Ltd. and The Distillers Co. Ltd. A phosphate buffer, pH 6.8 (equal vol. Na_2HPO_4 and NaH_2PO_4 , 0.2M), was used unless otherwise stated. The phosphoric-tungstic acid solution was prepared by dissolving 6 g. $Na_2WO_4 \cdot 2H_2O$ in 1 l. 0.15M- H_3PO_4 .

Extracts. The animal was killed by a sharp blow on the back of the neck and immediately bled. The liver was removed, weighed and ground with acid-washed sand (British Drug Houses Ltd.) and water (1.5 × the original weight). The resulting brei was centrifuged and the supernatant liquid decanted and used immediately.

Digests. NaCl (55 mg./100 ml.) was dissolved in phosphate buffer to ensure that the concentration of Cl^- in the final digest was not less than 3 mM. Chloro compound, to give the required final concentration (usually 0.024M), was added to a mixture of the buffer (15 ml.) and extract (10 ml.). Three drops of toluene were added as preservative. (Control experiments had shown that, up to 7 hr., toluene had no significant effect upon the liberation of Cl^- .) Chloro compounds insoluble in the mixture were dissolved in ethanol (2.5 ml.) and the volume of buffer reduced to 12.5 ml. As will be seen later, ethanol causes slight inhibition of the liberation of Cl^- from chloral hydrate. The digests were incubated at 37.4° in stoppered flasks. Control solutions were: (a) the digest with the extract replaced by a solution of NaCl (70 mg./100 ml.), and (b) the digest without chloro compound. The first gave a measure of the spontaneous liberation of Cl^- . A control with boiled extract was not used for the reason stated below. The usual concentration of SH compounds was 0.02M and when these were used, control digests without chloro compound were set up to give some indication of the extent of loss of SH-groups due to atmospheric oxidation, since it was impracticable to carry out the digestions, withdrawal of samples and analyses in an atmosphere of N_2 .

Estimation of chloride ions. The method finally adopted was essentially a modification of that described by Van Slyke & Hiller (1946), which was effective with concentrations of Cl^- over 3 mM. Digest (2 ml.) was thoroughly mixed with phosphoric-tungstic solution (5 ml.) and $AgIO_3$ (60 mg.) and centrifuged. The supernatant liquid was

separated, filtered and titrated (2 ml. portions with starch as indicator) against 0.01N- $Na_2S_2O_3$ immediately after addition of 200 mg. NaI. This method gave good results in the absence of tissue extracts, but recovery of added Cl^- tended to be low by up to 5% in the presence of extracts. The complete liberation of one Cl atom from 1 mol. substrate under the above conditions should cause an increase in titre of 8.21 ml. $Na_2S_2O_3$. Calculations have been based upon the assumption that this titre represents 100% liberation of Cl^- . Values significantly greater than 100% thus indicate the liberation of more than one Cl atom/mol. substrate. Under the conditions used, added SH compounds did not interfere, provided that titrations were carried out within 20 min. of the addition of $AgIO_3$. In some experiments the results by this method were confirmed by a polarographic method based upon that of Zimmerman & Layton (1949). For these, trichloroacetic acid solution (5%, w/v) was used in place of the phosphoric-tungstic acid solution.

Estimation of SH content. The method was based on those of King, Baumgartner & Page (1930), Kühnau (1931) and Lucas & King (1932). Digest (2–5 ml. according to SH content) was added to trichloroacetic acid (10%, w/v) to give total vol. 20 ml. The protein precipitated was removed by centrifugation (1 min. at 3000 rev./min.) followed by filtration. Filtrate (10 ml.) was cooled to 0° and mixed with 1 ml. KI (10% w/v) and 3 ml. 0.01N- I_2 . Excess I_2 was titrated with 0.002N- $Na_2S_2O_3$ using starch as indicator. The I_2 consumption was referred to calibration curves from which the SH content was calculated. Calibration curves were prepared from buffered (pH 6.8) NaCl solution of the SH compounds concerned in concentrations up to 0.026M. Glutathione (0.006M) was used for the calibration curve when cystine was added to digests. Confirmation of the presence and disappearance of SH groups was obtained by the nitroprusside test (Hopkins, 1921).

Estimation of urease. The method was essentially that of Marshall (1913), using urease tablets (British Drug Houses Ltd.) as the source of urease.

Estimation of papain. The milk-clotting method of Balls & Hoover (1937) was modified by mixing the enzyme with buffer (pH 4.6) and using fresh cow's milk (10 ml.) in place of the dried-milk solution.

RESULTS

Chloral hydrate and trichloroacetamide

Chloral hydrate (2:2:2-trichloro-1:1:1-dihydroxyethane) and trichloroacetamide were selected as substrates for the initial experiments. The former liberates chloride ions spontaneously, but the latter is stable in solution at pH 6.8 in the absence of liver extract.

Activity of liver extracts of various species. Digests were prepared with extracts of rabbit, guinea pig and rat liver. Owing to the small size, rat-liver extract was made with 2.5 parts water. The digests were incubated for 24 hr., samples being withdrawn at intervals. The results are shown in Table I, which also shows the extent of the spontaneous liberation of chloride ions from chloral hydrate. Trichloroacetamide digests included ethanol (10%, v/v).

Table 1. *Liberation of chloride ions from chloral hydrate and trichloroacetamide caused by liver extracts*

(Substrates 0.024M, pH 6.8. Results expressed as the percentage of 1 Cl/mol. Ranges are given in parentheses below the average values.)

age values.)		Percentage of chloride liberated				
Source of liver extract	No. of samples tested	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Chloral hydrate						
Rabbit	19	2.9 (1.0-5.2)	4.7 (2.1-7.3)	7.2 (4.1-9.7)	11.5 (7.2-16.1)	14.9 (9.8-20.7)
Guinea pig	6	2.7 (1.2-4.1)	4.8 (2.3-6.7)	8.0 (4.5-10.1)	13.9 (9.3-16.3)	18.0 (13.0-19.9)
Rat	6	1.1 (0.8-1.4)	1.7 (1.4-2.3)	2.8 (2.3-3.9)	4.9 (3.9-6.9)	6.7 (4.8-9.2)
None	7	0.2 (0.2-0.3)	0.4 (0.3-0.5)	0.8 (0.7-0.9)	1.9 (1.5-2.2)	4.6 (3.5-5.4)
Trichloroacetamide						
Rabbit	6	1.2 (0.9-1.5)	2.4 (1.7-3.0)	4.4 (2.9-5.5)	9.1 (5.2-11.6)	13.1 (7.3-16.8)

Effect of pH. Digests with chloral hydrate were made up with phosphate buffer of pH varying from 6.2 to 8.0. Liberation of chloride ions in the former was small, but an increased liberation at pH 8.0 was largely due to spontaneous decomposition of the chloral hydrate. Results from a typical experiment

with chloral hydrate are shown in Fig. 2 and similar results were obtained with trichloroacetamide in digests including ethanol (10%, v/v). Digests which liberated 8% chloride after 24 hr. with unboiled extract, liberated 6% when the extract had been previously boiled.

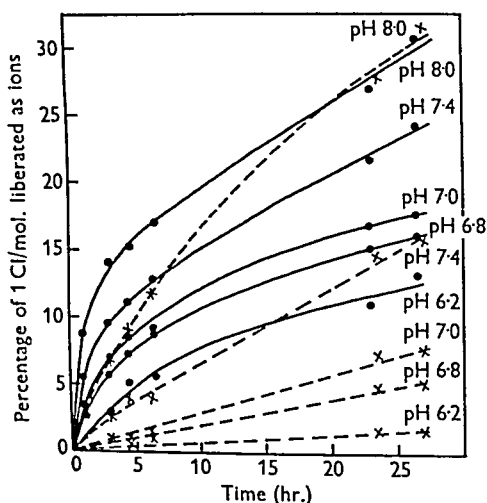


Fig. 1. The effect of pH upon the liberation of chloride ions from chloral hydrate (0.024M) by rabbit-liver extract (unbroken lines). Spontaneous liberation of chloride ions in absence of extract shown by broken lines.

is shown in Fig. 1. It is clear that the pH (6.8) selected for routine use is one at which liver extract uses appreciable liberation of chloride ions, whilst the extent of spontaneous decomposition is low.

Effect of boiling. Controls with boiled extracts were not used, since the extracts showed considerable activity even after boiling 1 hr. under reflux. The coagulum formed on boiling was finely ground before incubation.) The results of a typical experi-

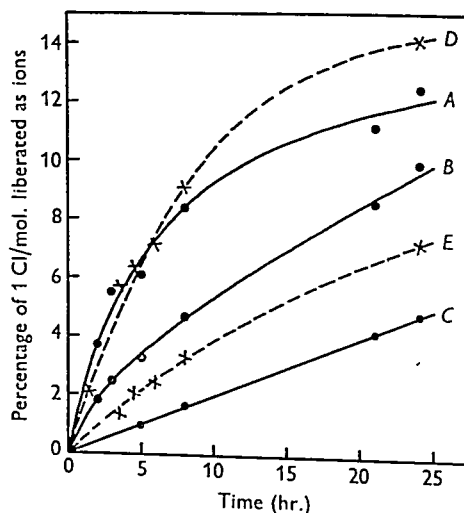


Fig. 2. The effect of boiling (unbroken lines) and of dialysis (broken lines) upon the chloride-liberating activity of rabbit-liver extract. Substrate, chloral hydrate (0.024M) at pH 6.8. Curve A, digest with untreated extract. Curve B, digest with the extract previously boiled 1 hr. Curve C, spontaneous liberation of chloride ions from chloral hydrate. Curve D, digest with another untreated extract. Curve E, digest with this extract previously dialysed 20 hr. against running water.

Effect of dialysis. Extracts were dialysed against running water for 20 hr. and their activity compared with that of the original extract which had been kept at the same temperature. In every case some loss of activity was observed, but the dialysed

extract always retained some activity. The results of an experiment with chloral hydrate are shown in Fig. 2. Similar results were obtained with trichloroacetamide, about half of the chloride-liberating activity of the extract being retained after dialysis.

Effect of ethanol. Since many of the chloro compounds used were sparingly soluble in the absence of ethanol, the effect of the addition of ethanol to chloral hydrate digests was examined. It is clear from Table 2 that the inclusion of ethanol (10% of total vol.) caused some inhibition of the

Table 2. *Liberation of chloride ions from chloral hydrate caused by rabbit-liver extracts with and without the addition of ethanol*

(Chloral hydrate 0.024M, ethanol 10% of total volume, pH 6.8. Results expressed as the percentage of 1 Cl/mol. Ranges are given in parentheses below the average values.)

Digest	No. of samples tested	Percentage of chloride liberated				
		1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Extract + chloral hydrate	7	3.1 (2.1-4.1)	5.1 (3.8-6.1)	8.1 (6.3-9.2)	13.2 (9.9-16.1)	17.3 (13.6-20.7)
Extract + chloral hydrate + ethanol	8	2.6 (1.1-4.2)	4.2 (2.1-6.5)	6.5 (3.7-9.4)	10.2 (6.3-14.1)	13.0 (9.4-17.9)
Chloral hydrate	7	0.2 (0.2-0.3)	0.4 (0.3-0.5)	0.8 (0.7-0.9)	1.9 (1.5-2.2)	4.6 (3.5-5.4)
Chloral hydrate + ethanol	4	0.2 (all 0.2)	0.4 (0.3-0.5)	0.8 (0.7-0.9)	1.9 (1.7-2.1)	4.6 (4.0-5.0)

Table 3. *Liberation of chloride ions from various chloro compounds by rabbit-liver extracts*

(Substrates normally 0.024M in 10% ethanol, pH 6.8. Results expressed as a percentage of 1 Cl/mol.)

Substrate	Percentage of chloride liberated									
	With extract					Without extract				
	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Chloroform	0.6	1.1	2.0	3.9	6.4	0.0	0.1	0.2	0.4	1.2
Benzyl chloride	9.3	14.8	22.0	35.1	52.8	4.6	8.4	14.3	26.2	46.9
Benzotrichloride	23.5	38.1	62.5	120.2	193.1	28.7	46.9	71.9	108.0	159.8
Ethylene dichloride	0.0	0.0	0.1	0.3	0.8			None		
(1:2-Dichloroethane)*										
1:1:2:2-Tetrachlorethane	0.7	1.3	2.4	5.4	12.6	0.2	0.4	0.8	2.1	5.2
Pentachloroethane	2.0	3.7	6.7	15.1	31.9	1.1	2.1	3.8	7.1	13.0
Hexachloroethane	2.3	4.1	7.0	13.5	23.3			None		
Trichloroethylene	0.0	0.1	0.2	0.5	1.1			None		
Ethylene chlorohydrin	0.3	0.5	0.8	1.4	2.3	0.0	0.1	0.2	0.5	1.2
(2-Chloroethanol)†										
Trichloroacetic acid‡	1.1	1.8	3.0	4.9	6.9			None		
Trichloroacetamide	1.4	2.8	5.3	11.0	16.8			None		
Chloral hydrate†	4.1	5.9	8.1	12.3	15.0	0.2	0.4	0.8	1.9	4.6
Chloralose	1.1	2.2	4.1	7.9	11.3			None		
ω-Chloroacetophenone	4.5	7.5	13.0	25.1	43.2	0.1	0.2	0.4	1.1	2.5
1:2-Dichloropropane	0.2	0.4	0.6	1.3	2.0	0.0	0.1	0.2	0.5	1.1
1-Chloropropionic acid‡	1.1	1.9	2.9	4.6	6.2	0.1	0.2	0.4	0.9	2.1
2-Chloropropionic acid‡	2.3	3.7	5.6	9.0	11.6	0.4	0.8	1.6	4.1	9.6
1:3-Dichloroacetone†	40.8	52.7	70.0	101.5	127.8	9.9	19.7	40.0	85.6	130.1
Chloretone	0.9	1.6	2.8	6.2	13.3	0.1	0.2	0.4	0.9	2.3
Butyl chloral hydrate	13.1	25.3	36.1	45.9	49.6	0.2	0.4	0.7	1.9	4.5
iso-Amyl chloride§	0.1	0.2	0.2	0.3	0.4			None		
(1-Chloro-3-methylbutane)										
Chlorobenzene	0.5	0.6	0.9	1.4	1.9			None		
p-Dichlorobenzene	0.9	1.4	1.9	2.3	2.8			None		
o-Chlorobenzoic acid‡	0.4	0.6	0.8	1.3	1.5			None		
m-Chlorobenzoic acid‡	1.1	1.9	2.5	3.1	3.3			None		
p-Chlorobenzoic acid‡	0.8	1.3	1.7	2.0	2.1			None		
o-Chlorobenzamide	0.3	0.5	0.9	1.7	2.7			None		
m-Chlorobenzamide	0.1	0.2	0.3	0.8	1.6			None		
p-Chlorobenzamide	0.3	0.5	0.9	1.7	2.8			None		

* 0.036M.

† Substrate made up with 1 equiv. NaHCO₃.

‡ Digests did not contain ethanol.

§ 0.072M.

liberation of chloride ions, although it had no effect upon the spontaneous decomposition of chloral hydrate.

Other chloro compounds

Rabbit-liver extract was used in the examination of twenty-seven other chloro compounds. In every experiment a digest with chloral hydrate was used to confirm the activity of the liver extract. Typical results for each of the twenty-nine compounds used

hydrate upon guinea pigs and rats. Six animals were given thirteen injections of chloral hydrate (25 mg.) subcutaneously on alternate days. The animals were then killed and the activity of the livers compared with those of six animals kept as controls. The activity of the livers of the treated animals was slightly less than those of the control animals. In no experiment was increased activity observed. A typical experiment with guinea pigs (350–450 g. body weight) is shown in Table 4.

Table 4. *Effect of previous injection of guinea pigs with chloral hydrate upon the liberation of chloride ions from chloral hydrate caused by extracts of their livers*

(Substrates 0.024M, pH 6.8. Results expressed as percentage of 1 Cl/mol. Ranges are given in parentheses below the average values. There were six animals in each group.)

Source of liver extract	Percentage of chloride liberated				
	1 hr.	2 hr.	4 hr.	10 hr.	24 hr.
Injected animal	2.6 (1.4–4.4)	4.2 (2.6–6.1)	6.5 (4.6–8.7)	11.0 (8.5–13.8)	15.3 (11.7–18.6)
Untreated animals	2.7 (1.2–4.1)	4.8 (2.3–6.7)	8.0 (4.5–10.1)	13.9 (9.3–16.3)	18.0 (13.0–19.9)

are given in Table 3. It is clear that the extent of liberation of chloride ions by liver extract is not directly related to the ease of spontaneous liberation of chloride ions from a compound. Hexachloroethane, trichloroacetamide and chloralose (1-(2':2':2'-trichloro-1'-hydroxyethyl)-1:6-anhydroglucopyranose), yield a considerable percentage of their chlorine as ions with tissue extract but none spontaneously. Some compounds, butylchloral hydrate (2:2:3-trichloro-1:1-dihydroxybutane), ω -chloroacetophenone, chloral hydrate and chloretone (1:1:1-trichloro-2-hydroxy-2-methylpropane), yield small amounts of chloride ions spontaneously but much more in the presence of tissue extract. Other compounds which readily liberate considerable amounts of chloride spontaneously, benzyl chloride, benzotrichloride (ω -trichlorotoluene), and penta-chloroethane, liberate still more with tissue extract. 1:3-Dichloroacetone, which liberates more than half its chlorine as ions spontaneously, does not liberate any more after 24 hr. in the presence of liver extract, although the extract accelerates the initial liberation of chloride ions. Several compounds, including those in which chlorine is directly attached to the benzene ring, liberate no significant amount of chloride either spontaneously or with liver extract.

Activity of liver extracts after repeated injection of chloral hydrate

Heppel & Porterfield (1948) observed that the livers of rats which had been repeatedly exposed to an atmosphere containing bromochloromethane caused a greater increase in liberation of bromide ions than the livers of untreated animals. Experiments with this object were carried out with chloral

Experiments with SH compounds

When chloral hydrate is incubated with liver extract there is in addition to the liberation of chloride ions, a loss in the SH content of the digest. This can be seen from the results recorded in Table 5 which gives the liberation of chloride ions and the loss of SH in m-equiv./l. digest. While the differences between the chloride values give the increased liberation of chloride ions due to reaction of chloral hydrate with extract, the differences between SH values are unlikely to give a measure of the SH compounds combining with chloral hydrate since the reaction with chloral is obviously very rapid compared with the reactions (e.g. atmospheric oxidation) causing disappearance of SH from extracts in the absence of chloral hydrate. For example 40 % of the total SH is lost in the first hour when chloral hydrate is present, but there is no detectable loss in the absence of chloral hydrate. It should also be remembered that the method for SH estimation determines only SH soluble in trichloroacetic acid and that any SH groups bound to protein would be precipitated and removed before titration. The results do, however, indicate that the liberation of chloride ions involves a loss in the SH content of the extract.

The effect of the addition of cysteine. It can be seen from the results of a typical experiment in Fig. 3 that cysteine in the absence of liver extract causes liberation of chloride ions and that if liver extract is also present the chloride liberation is much greater than when either extract or cysteine alone is present. Table 6 summarizes the results of some experiments with chloral hydrate and cysteine with

Table 5. *Average liberation of chloride ions from chloral hydrate and the average loss in SH content of rabbit-liver extract when incubated together*

(Chloral hydrate 0.024M, pH 6.8, 37°. Results expressed in m-equiv./l. digest and ranges given in parentheses.)

Digest	No. of samples tested		1 hr.	2 hr.	4 hr.	10 hr.	Initial SH content
Extract + chloral hydrate	16	Cl ⁻ liberated	0.9 (0.5-1.1)	1.4 (0.9-1.8)	2.1 (1.3-2.5)	3.2 (2.3-3.6)	—
Chloral hydrate alone	4	Cl ⁻ liberated	0.0 —	0.1 (0.1)	0.2 (0.1-0.3)	0.5 (0.4-0.7)	—
		Difference	0.9	1.3	1.9	2.7	—
Extract + chloral hydrate	8	SH lost	0.9 (0.1-1.4)	1.2 (0.2-1.8)	1.5 (0.5-2.2)	1.7 (0.9-2.5)	2.3 (1.3-2.9)
Extract alone	8	SH lost	0.0 (+0.4*-0.3)	0.2 (+0.4*-0.5)	0.6 (0.1-0.9)	1.2 (0.6-1.8)	2.3 (1.3-2.9)

* + indicates a gain in SH content.

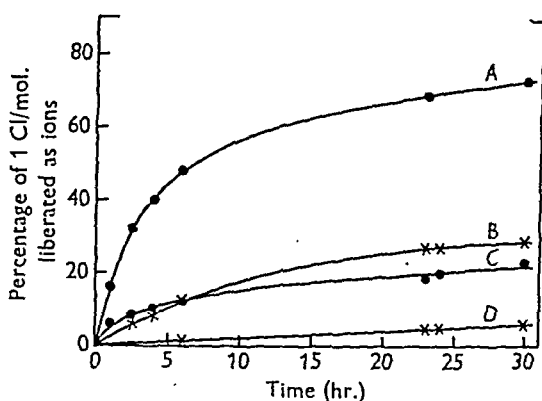


Fig. 3. The effect of cysteine (0.02M) upon the liberation of chloride ions from chloral hydrate (0.024M) by rabbit-liver extract at pH 6.8. Curve A, digest with chloral hydrate, extract and cysteine. Curve B, digest with chloral hydrate and cysteine. Curve C, digest with chloral hydrate and extract. Curve D, spontaneous liberation of chloride ions from chloral hydrate at pH 6.8.

Table 6. *Average liberation of chloride ions from chloral hydrate and average loss in SH content of cysteine when incubated with and without rabbit-liver extract*

(Chloral hydrate 0.024M, cysteine 0.02M, pH 6.8 at 37°. Results expressed in m-equiv./l. digest and ranges given in parentheses.)

Digest	No. of samples tested		1 hr.	2 hr.	4 hr.	10 hr.	Initial SH content
Cysteine + chloral hydrate	6	Cl ⁻ liberated	0.6 (0.5-0.8)	1.2 (0.9-1.4)	2.2 (1.7-2.5)	4.7 (3.7-5.0)	—
Chloral hydrate alone	4	Cl ⁻ liberated	0.0	0.1 (0.1)	0.2 (0.1-0.3)	0.5 (0.4-0.7)	—
		Difference	0.6	1.1	2.0	4.2	—
Cysteine + chloral hydrate	4	SH lost	1.1 (0.4-1.6)	1.7 (1.0-2.4)	3.4 (2.5-4.0)	8.6 (8.5-9.1)	20.3 (18.9-22.5)
Cysteine alone	4	SH lost	0.5 (0.2-0.6)	1.0 (0.7-1.2)	2.1 (1.6-2.4)	5.5 (4.9-6.2)	20.2 (18.9-22.5)
		Difference	0.5	0.7	1.3	3.1	—
Extract + cysteine + chloral hydrate	3	Cl ⁻ liberated	3.2 (2.8-3.7)	5.8 (5.0-6.3)	9.2 (7.9-10.0)	13.6 (12.6-14.8)	—
		Difference*	3.2	5.7	9.0	13.1	—
Extract + cysteine + chloral hydrate	3	SH lost	3.9 (2.5-4.6)	6.4 (4.5-7.5)	10.0 (7.6-11.8)	15.2 (13.7-16.7)	20.5 (19.6-21.0)
Extract + cysteine	3	SH lost	1.0 (0.7-1.4)	1.9 (1.4-2.7)	3.6 (2.9-4.6)	8.3 (7.6-9.5)	20.5 (19.6-21.0)
		Difference	2.9	4.5	6.4	6.9	—

* By subtracting 'chloral hydrate alone' values (see text).

and without liver extract in which both chloride liberation and SH loss have been determined. Again it is seen that the liberation of chloride ions involves a loss of SH. For the experiments in the presence of extract the values for chloride liberated, corrected for spontaneous liberation of chloride ions (i.e. by subtraction of the 'chloral hydrate alone' values), represent the liberation of chloride due to extract + cysteine. By subtracting the SH lost for 'extract + cysteine' from that for 'extract + cysteine + chloral hydrate', values should be obtained which represent the SH loss from extract + cysteine concerned in the liberation of chloride ions from chloral hydrate. This difference, therefore, should be comparable with the corrected values for chloride liberation. The assumption that the loss of SH due to oxidation (or other unknown reactions) is the same in the presence or absence of chloral hydrate is not, however, justified since oxidation would almost certainly be greater in the absence of chloral when there is no competition with the chloride-SH reaction. The difference values would thus be expected to be low, and it may reasonably be concluded that both with and without liver extract there is a loss of SH corresponding fairly closely with the liberation of chloride ions.

Chloral hydrate and other SH compounds. Experiments similar to those described above were carried out with other SH compounds. From these only the average values (from at least three experiments) corresponding to the 'differences' in Table 6 have been recorded in Table 7 and only 4 hr. values are given. The loss of SH with glutathione and extract was greater than that expected from the chloride ions liberated. With thioglycollate and extract the SH loss was also somewhat greater than the chloride liberation, although there was no loss of SH when chloride ions were liberated in the absence of extract.

Other chloro compounds with cysteine. With butyl chloral hydrate in the presence of extract the loss in SH was approximately equivalent to chloride liberated, but in the absence of extract the SH loss was greater than that equivalent to the chloride liberated (Table 7). With trichloroacetamide, both with and without extract, the SH loss was much less

than would have been expected from the amount of chloride liberated.

Chloral hydrate and cystine. In experiments in which cystine (0.02M) was added to liver extracts with chloral hydrate there was a significant increase in chloride liberation (1.1 m-equiv./l. digest in 4 hr.) over that obtained from extract and chloral hydrate alone (2.1 m-equiv./l.). There was no increase in the absence of extract. When cystine was added to extract without chloral hydrate it was found that there was almost always an increase in SH content (average 0.3 m-equiv./l. in 4 hr.). This increase may explain the effect of cystine upon the activity of liver extract. In the presence of chloral hydrate there was a loss in SH corresponding to 0.7 m-equiv./l.

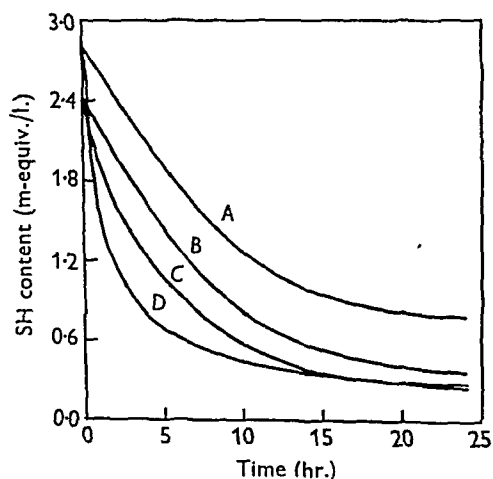


Fig. 4. The effect of previous boiling (1 hr.) upon the SH content of rabbit-liver extract incubated with and without chloral hydrate (0.024M) at pH 6.8. Each curve represents average results from three experiments. Curve A, untreated extract. Curve B, boiled extract. Curve C, boiled extract with chloral hydrate. Curve D, untreated extract with chloral hydrate.

Effect of boiled liver extract. It has already been stated that considerable liberation of chloride ions from chloral hydrate occurs in the presence of boiled extract. The average results for utilization of SH from three experiments are summarized in Fig. 4.

Table 7. Average liberation of chloride ions from chloro compounds and the corresponding loss in SH content due to incubation with SH compounds with and without rabbit-liver extract

(Chloro compound 0.024M, SH compound 0.02M, pH 6.8, 37°, 4 hr. incubation. Results expressed as m-equiv./l.)

Substrates	Without liver extract		With liver extract	
	Cl ⁻ liberated	SH loss	Cl ⁻ liberated	SH loss
Chloral hydrate with:				
Cysteine	2.0	1.3	9.0	6.4
Glutathione	1.8	1.7	4.5	6.5
Thioglycollate	3.1	0.0	4.0	4.6
Butyl chloral hydrate with cysteine	1.3	1.7	7.3	6.4
Trichloroacetamide with cysteine	2.5	0.5	1.8	0.5

Boiling causes a reduction in the SH content of the extract, but even with boiled extract there is a more rapid disappearance of SH with chloral hydrate than with boiled extract alone.

Effect of chloral hydrate upon the activity of urease. Urease is an SH enzyme and should be inhibited by a substance such as chloral hydrate which reacts with SH groups. Urease was incubated with chloral hydrate for periods up to 3 hr. prior to determination of the urease activity of the digest. The results recorded in Table 8 show that treatment with chloral hydrate causes considerable inhibition of urease.

chloride ions by tissue extracts. These compounds are either aliphatic, or the chlorine is attached to an aliphatic side chain. In no instance was there appreciable liberation of chloride ions from nuclear-substituted aromatic compounds. It is far from certain, however, that the liberation of chloride from the aliphatic compounds is due to an enzyme such as that claimed by Heppel & Porterfield (1948) to liberate halide from some halogenated hydrocarbons. The greater part of the factor responsible is remarkably stable to heat. Only part of the activity of a liver extract is retained after dialysis. It has been shown that chloride ions can readily be liber-

Table 8. *Effect of preliminary incubation with chloral hydrate upon the activity of urease*
(Results expressed as percentage conversion of urea, 0.9 g./l.)

Exp. no.	Time of preliminary incubation (hr.)	Concn. of chloral hydrate (M)	Percentage of urea converted				
			0.5 hr.	1 hr.	1.5 hr.	2 hr.	2.5 hr.
1	0	0	44	72	86	91	91
		0.024	31	41	57	63	66
2	0	0	41	69	85	91	93
		0.096	23	30	33	34	34
3	1	0	40	69	83	—	92
		0.096	11	16	19	—	20
4	2	0	30	52	67	—	82
		0.096	5	7	8	—	9
5	3	0	26	43	54	61	67
		0.096	3	5	6	7	7

Table 9. *Effect of preliminary incubation for 30 min. with chloro compounds upon the clotting power of papain*

Exp. no.	Chloro-compound	Amount of papain (mg./10 ml. milk)						
		20	24	28	30	32	36	40
		Reciprocal of clotting time (min. ⁻¹)						
1	None	0.8	0.9	1.0	1.2	1.5	1.7	—
	Chloral hydrate (0.02M)	0.5	0.7	0.7	0.9	1.0	1.2	—
2	None	—	1.0	1.5	1.6	1.7	2.1	2.2
	Chloral hydrate (0.04M)	—	0.7	0.8	0.9	1.1	1.3	1.5
3	None	—	0.8	0.9	1.2	1.3	1.6	1.8
	Butyl chloral hydrate (0.01M)	—	0.8	0.8	0.9	0.9	1.2	1.5

Effect of chloral hydrate upon the activity of papain. Another SH enzyme, papain, was incubated with chloro-compound for 30 min. previous to determination of activity in clotting of milk. As with urease there was considerable loss of activity. Typical results are shown in Table 9 which records the reciprocal of the time in minutes taken to clot milk using varying amounts of papain.

DISCUSSION

It is clear from the experiments described that a considerable percentage of the chlorine of certain organic chloro compounds may be liberated as

ated from chloral hydrate in the absence of liver extract if cysteine is present, and that in the presence of liver extract the addition of cysteine enhances the liberation of chloride ions. The experimental results suggest a quantitative relationship between the chloral hydrate and the SH groups of cysteine and/or liver extract, although such a relationship was not always clear when other chloro or SH compounds were used. It must, however, be remembered that the method of estimation of SH content did not permit the estimation of SH groups bound to protein, and that SH groups may also be used up in reactions, other than atmospheric oxidation, not involving the chlorine atoms, e.g. by condensation

between SH and aldehyde group (cf. Schubert, 1936). Such reactions could account for a loss in the SH content of the digest greater than that expected from the chloride ions liberated, e.g. from chloral hydrate with glutathione or thioglycollic acid (Table 7). It seems probable that the action of liver extract in liberating chloride from chloro compounds is for the most part not enzymic but due to chemical reaction between the chloro compound and SH compounds in the liver extract. If there is a chloride-liberating enzyme it can only play a very small part in the reactions which have been studied. The fact that the addition of the disulphide cystine causes some enhancement of liberation of chloride ions in the presence of liver extract suggests that the extract can reduce disulphide compounds and so enable them to react with chloro compounds.

SUMMARY

1. The effect of incubation with tissue extracts upon the liberation of chloride ions from twenty-nine organic chloro compounds has been examined.
2. Some compounds, including those in which the chlorine is directly attached to the benzene ring, do not liberate appreciable amounts of chloride. Some

aliphatic chloro compounds which liberate little chloride, e.g. chloral hydrate, butyl chloral hydrate, or none, e.g. trichloroacetamide, hexachloroethane, spontaneously in aqueous solution, liberate considerable amounts in the presence of liver extract; others which liberate considerable amounts of chloride spontaneously, e.g. benzotrichloride, benzyl chloride, liberate even more in the presence of liver extract.

3. The loss in SH content of the extract always observed when chloride ions are liberated suggests that the reaction is largely one between the chlorine of the chloro compound and the SH of SH compounds.

4. The liberation of chloride ions is enhanced by the addition of SH compounds both in the presence and absence of liver extracts.

5. Previous boiling does not greatly diminish the chloride-liberating activity of liver extracts.

6. It is concluded that the liberation of chloride ions in the presence of liver extract is mainly a reaction between chloro compound and SH compounds which is not enzymic.

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Studies in Detoxication

43. A STUDY OF THE ARYLSULPHATASE ACTIVITY OF TAKADIASTASE TOWARDS SOME PHENOLIC ETHEREAL SULPHATES

By D. ROBINSON, J. N. SMITH, B. SPENCER AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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Several types of sulphatases occur in nature. These are distinguished by the nature of the ester group found in the ethereal sulphates which they hydrolyse (Fromageot, 1950). Takadiastase, a commercial preparation of *Aspergillus oryzae*, possesses arylsulphatase (phenolsulphatase) activity (Neuberg & Kurono, 1923), for it hydrolyses phenolic monoesters of sulphuric acid but has no action on aliphatic sulphuric esters. Others preparations of *A. oryzae* such as 'Mylase P' (Abbott, 1947) and 'Clarase' (Dzialoszynski, 1947) are also active. Arylsulphatase also occurs in animal tissues, especially liver, and according to Huggins & Smith (1947) rat neoplasms contain significantly higher amounts of the enzyme than normal tissues.

Mylase P has been shown to release phenolic oestrogens from their conjugates in pregnant mares' urine in yields 20% higher than those obtained by acid hydrolysis (Cohen & Bates, 1949). The use of sulphatase thus provides a gentle method of hydrolysis for ethereal sulphates where acid hydrolysis might prove destructive. We required sources of arylsulphatase for this purpose, and the present work was undertaken to provide possible methods of assay of arylsulphatase and to obtain information about its activity towards different phenolic sulphuric esters.

Recent methods of arylsulphatase assay are those of Abbott (1947) using potassium phenylsulphate with the Folin-Ciocalteu reagent, and of Huggins & Smith (1947) using potassium *p*-nitrophenylsulphate as substrate (cf. Abbott & East, 1949). In the present work we have compared the action of takadiastase on the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids. In each case the liberated phenols were estimated in alkaline solution as their anions whose spectra were different from the corresponding undissociated phenols and ethereal sulphates. By this method each phenol could be estimated spectrophotometrically or colorimetrically in the presence of its ethereal sulphate, using a relatively short incubation time in the enzyme experiments.

EXPERIMENTAL

Spectroscopic measurements were made with a Unicam quartz spectrophotometer, Model SP500, colorimetric measurements with a Hilger Spekker absorptiometer and pH measurements with a Cambridge pH-meter.

Preparation of substrates. The potassium salts of *o*- and *p*-nitro-, *p*-chloro- and *p*-aldehydo-phenylsulphuric acids and the sodium salt of *m*-nitrophenylsulphuric acid were prepared according to Burkhardt & Lapworth (1926) and Burkhardt & Wood (1929). The potassium salts of 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids were prepared for the first time by one of us (Smith, 1951). These salts were purified by recrystallization from water or ethanol-water mixtures until free from inorganic sulphate, chloride and free phenols. Their purity was checked by analysis for Na or K gravimetrically, or for phenols spectroscopically.

Absorption spectra of the substrates and phenolic anions. These were determined in 0.1N-NaOH and are recorded in Table 1. Fig. 1 shows the spectra of 4-nitrocatechol and its monosulphate and illustrates the difference between the spectrum of a phenolic anion and that of its sulphate.

Preparation of the enzyme solution. Takadiastase (Parke, Davis and Co. Ltd.) was either dissolved in water, or in 0.5M-acetate buffer as required. These solutions were kept at 0°, but they deteriorated in about a week. The takadiastase used contained added lactose (reducing sugar 96%, calc. as lactose) and 0.4% N (Kjeldahl). This commercial preparation was thus largely lactose. It has been reported that phosphates inhibit sulphatase and therefore phosphate buffers were avoided. At the optimum pH of sulphatase (about pH 6), acetate buffers are not highly efficient, so that they were used at 0.5M strength to ensure their effectiveness.

The action of takadiastase on p-aldehydo- and p-chloro-phenylsulphates

Methods

(a) *Using potassium p-aldehydophenylsulphate.* To each of four tubes there were added 0.4 ml. of 0.5M-acetate buffer and 0.4 ml. enzyme solution in 0.5M-acetate buffer. To two of these tubes ('test') there was added 0.4 ml. of a solution of potassium *p*-aldehydophenylsulphate in 0.5M-acetate buffer of the same pH (all solutions having been previously warmed to 37°). The other two tubes were the controls. All tubes were stoppered and incubated for 1 hr. at 37°. After incubation, 0.4 ml. of substrate solution was added to the

Table 1. Absorption maxima of certain phenols and their ethereal sulphates in 0.1N-NaOH

	$\lambda_{\max.}$	$\epsilon_{\max.}$	$\lambda_{\max.}$	$\epsilon_{\max.}$
<i>o</i> -Nitrophenol	283	3850	412-415	4500
Potassium <i>o</i> -nitrophenylsulphate	260	4400	—	—
<i>m</i> -Nitrophenol	290	4050	395	1450
Sodium <i>m</i> -nitrophenylsulphate	265	6500	—	—
<i>p</i> -Nitrophenol	230	4550	402	17 000
Potassium <i>p</i> -nitrophenylsulphate	280	9000	—	—
<i>p</i> -Chlorophenol	222	8900	245	11 650
			298	2600
Potassium <i>p</i> -chlorophenylsulphate	215	10 500	267.5	350
			275	300
<i>p</i> -Hydroxybenzaldehyde	237	7680	330	27 500
Potassium <i>p</i> -aldehydophenylsulphate	258	16 300	—	—
Nitroquinol	237	13 100	540	3850
Potassium 4-hydroxy-2-nitrophenylsulphate	235	17 055	380	1390
Potassium 4-hydroxy-3-nitrophenylsulphate	225	16 800	423	4750
	279	3900	—	—
4-Nitrocatechol	390-395	6300	510	11 300
Potassium 2-hydroxy-5-nitrophenylsulphate	405-410	18 150	—	—

two control tubes and 3.8 ml. of 0.15N-NaOH to all four tubes. The extinctions of the tubes were then read at 330 $m\mu$. in the spectrophotometer, using 1 cm. cells, against a blank of 0.8 ml. buffer, 0.4 ml. water and 3.8 ml. 0.15N-NaOH. The amount of *p*-hydroxybenzaldehyde liberated was then calculated from the relation:

$$p\text{-hydroxybenzaldehyde } (\mu\text{g.}) = \frac{(E_t - E_c) \times 122.1 \times 10^4 \times 5}{27\,000 \times 100} \\ = 22.2 (E_t - E_c),$$

where E_t and E_c are the observed spectrophotometer readings of the 'test' and 'control' tubes respectively at 330 $m\mu$.; 122.1 is the molecular weight and 27 000 the ϵ_{\max} .

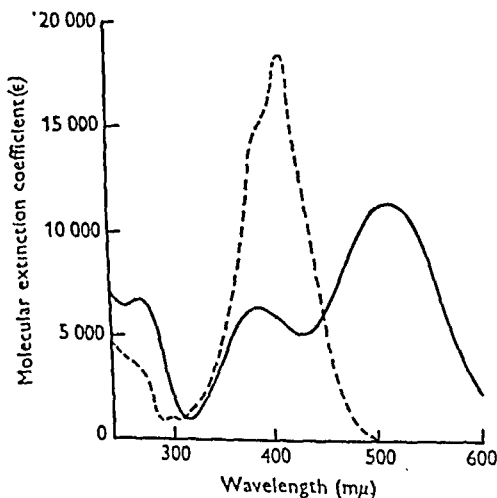


Fig. 1. The ultraviolet absorption spectra of 4-nitrocatechol (full line) and potassium 2-hydroxy-5-nitrophenylsulphate (broken line) in 0.1N-NaOH.

in alkali of *p*-hydroxybenzaldehyde. Control tubes of substrate and buffer were omitted because a separate experiment showed that no *p*-hydroxybenzaldehyde was liberated on incubating potassium *p*-aldehydophenylsulphate with acetate buffers from pH 4.1 to 6.7. The control tubes con-

taining enzyme and buffer, however, were included because the light absorption of the enzyme extracts may vary slightly on incubation with acetate buffer. At 330 $m\mu$. proteins absorb slightly, but in view of the very low protein content of takadiastase, the removal of protein was unnecessary. However, with preparations of arylsulphatase of high protein content, it may be necessary to remove protein as described in the next section.

(b) *Using potassium p-chlorophenylsulphate.* The procedure followed was similar to that described by Spencer & Williams (1951) for the assay of β -glucuronidase with *p*-chlorophenyl-glucuronide. In preliminary experiments it was found that potassium *p*-chlorophenylsulphate on incubation for 3 hr. with 0.5M-acetate buffers, pH 4.1-6.7, yielded no spectrophotometrically detectable amount of *p*-chlorophenol. The procedure for the assay of the arylsulphatase activity of takadiastase was the following.

The reagents and procedure up to the incubation were as in section (a) above except that centrifuge tubes were used. After incubation for 2 or 3 hr., 0.4 ml. of substrate solution was added to the control tubes, and then 4.8 ml. of absolute ethanol to all four tubes. The tubes were centrifuged for 3 min. to remove any precipitated protein and 5 ml. of the clear supernatants transferred to tubes containing 5 ml. 0.2N-NaOH. The extinctions of these solutions were then read at 245 $m\mu$. in the spectrophotometer against blanks made up of 0.4 ml. acetate buffer and 0.8 ml. water diluted with ethanol and 0.2N-NaOH as for the test solutions. The amount of *p*-chlorophenol liberated is

$$\frac{(E_t - E_c) \times 128.5 \times 6 \times 10\,000}{11\,650 \times 5} = 132(E_t - E_c) \mu\text{g.}$$

A study of the recovery of *p*-chlorophenol measured at 245 $m\mu$. and of *p*-hydroxybenzaldehyde at 330 $m\mu$., showed that the maxima appeared to obey the Lambert-Beer Law, and that the phenols could be recovered over a wide range of concentrations to the extent of $100 \pm 3\%$.

Effect of pH and substrate concentration on the hydrolysis of potassium p-aldehydo- and p-chloro-phenylsulphate by takadiastase is shown in Table 2.

In optimum conditions with these two substrates, extent of hydrolysis was proportional to time up to 4 hr. and to the quantity of takadiastase present up to 0.5%.

Table 2. *Investigation of effects of pH and substrate concentration on hydrolysis of substituted phenylsulphates by takadiastase*

(Temperature 37° throughout; other conditions noted in parentheses; taka = takadiastase.)

Substrate (all K salts unless otherwise stated)	Optimum pH	Optimum substrate concn. and K_m
<i>p</i> -Aldehydophenylsulphate	5.8 (little variation 5.5-6.1) (1% taka; 0.003M-substrate; 1 hr.)	$2.7 \times 10^{-3}M$ No inhibition up to $3 \times 10^{-2}M$ K_m , $2.1 \times 10^{-4}M$ (pH 5.8; 1% taka; 1 hr.)
<i>p</i> -Chlorophenylsulphate	6.15 (sharp maximum) (3% taka; 0.0125M-substrate; 3 hr.)	$1 \times 10^{-2}M$ No inhibition up to $3 \times 10^{-2}M$ K_m , $9.7 \times 10^{-4}M$ (pH 6.15; 3% taka; 3 hr.)
<i>o</i> -Nitrophenylsulphate	6.3 (little variation 6.0-6.5) (4% taka; 0.005M-substrate; 1 hr.)	$6 \times 10^{-3}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $3.4 \times 10^{-4}M$ (pH 6; 4% taka; 1 hr.)
<i>m</i> -Nitrophenylsulphate (Na salt)	6.2-6.4 (little variation 5.9-6.5) (4% taka; 0.005M-substrate; 2 hr.)	$6 \times 10^{-3}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $8.35 \times 10^{-4}M$ (pH 6; 4% taka; 2 hr.)
<i>p</i> -Nitrophenylsulphate	6.2 (little variation 6.0-6.4) (1% taka; 0.005M-substrate; 1 hr.)	$1.5 \times 10^{-2}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $1.7 \times 10^{-4}M$ (pH 6.1; 1% taka; 1 hr.)
4-Hydroxy-2-nitrophenylsulphate	6.0 (little variation 5.9-6.1) (2% taka; 0.002M-substrate; 2 hr.)	$2 \times 10^{-3}M$ No inhibition up to $5 \times 10^{-3}M$ K_m , $1.25 \times 10^{-4}M$ (pH 5.9; 2% taka; 2 hr.)
2-Hydroxy-5-nitrophenylsulphate	5.8-5.9 (2% taka; 0.0025M-substrate; 2 hr.)	$2.5 \times 10^{-3}M$ Inhibition beyond $2.5 \times 10^{-3}M$ K_m , $3.5 \times 10^{-4}M$ (pH 5.9; 2% taka; 2 hr.)

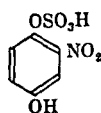
*The action of takadiastase on the o-, m- and p-nitrophenylsulphates**Method*

A solution (1 ml.) of the substrate dissolved in M-acetate buffer and the aqueous enzyme solution (1 ml.) were added to two tubes. These tubes, together with a control tube containing the substrate-buffer solution only, were incubated at 37° for the required period. Then 1 ml. of 0.33N-NaOH was added to all three tubes and 1 ml. of enzyme solution to the control tube. The extinctions of the contents of the first two tubes were then read in the spectrophotometer, with the contents of the control tube in the blank cell of the instrument. Readings were made at 412 m μ . for *o*-, at 395 m μ . for *m*- and at 402 m μ . for *p*-nitrophenol. The amount of nitrophenol in 3 ml. of test solution was then calculated from the relation, $\mu g.$ nitrophenol = Ex , where x is 92.66 for *o*-, 287.6 for *m*- and 24.5 for *p*-nitrophenol and E is the spectrophotometer reading at the appropriate wavelength, x being calculated as before from known ϵ_{max} and molecular weights of the phenols and the dilutions of the solutions. The maxima of each phenol appeared to obey the Lambert-Beer Law.

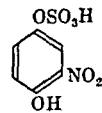
The effects of pH and of substrate concentration on the hydrolysis of the three nitrophenylsulphates are quoted in Table 2. In optimum conditions with these three substrates the extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4% for the *o*- and *m*-substrates and up to 1.2% for the *p*-substrate.

The action of takadiastase on certain hydroxynitrophenylsulphates

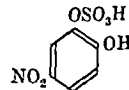
The two ethereal sulphates, 4-hydroxy-2-nitro- and 4-hydroxy-3-nitro-phenylsulphuric acids (I and II) give rise to nitroquinol on hydrolysis. Nitroquinol on making alkaline gives a purple colour (λ_{max} , 540 m μ ., ϵ_{max} , 3850) which can be estimated with the Spekker absorptiometer. The colour



I



II



III

fades on standing, but is sufficiently stable to allow readings of its intensity to be taken. Similarly, 2-hydroxy-5-nitrophenylsulphuric acid (III) gives rise to 4-nitrocatechol which gives a relatively stable red colour in alkaline solution (λ_{max} , 510 m μ ., ϵ_{max} , 11 300) which again can be estimated in the colorimeter. The alkaline nitrocatechol colour is more intense than that of nitroquinol and is therefore more suitable for the assay of arylsulphatase. The standard curves for nitroquinol and nitrocatechol were constructed by dissolving 10-70 $\mu g.$ of nitroquinol or 4-30 $\mu g.$ of 4-nitrocatechol in 2 ml. water and adding 0.5 ml. of 2.5N-NaOH. The intensity of colour varied linearly with the amount of the two phenols. The nitroquinol and nitrocatechol colours were read immediately in the Spekker absorptiometer with

Ilford no. 604 (Spectrum green) filters, and using 1 cm. cells holding 0.5 ml. of solution.

Method. The substrate solution (1 ml.), made by dissolving the potassium salt of the ethereal sulphate in M-acetate buffer, was added to each of three tubes. To two of the tubes 1 ml. of the aqueous enzyme solution was added and all three tubes were incubated at 37° for the required time. To each tube there was then added 0.5 ml. of 2.5 N-NaOH and to the third tube (control) 1 ml. of enzyme solution. The colours of the two test solutions were then measured in the Spekker absorptiometer with the control solution in the blank cell of the instrument. The amount of nitroquinol or nitrocatechol liberated was then read from the standard curves.

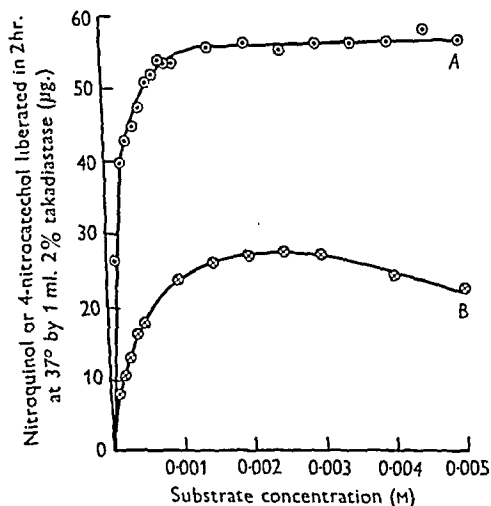


Fig. 2. The effect of substrate concentration on the arylsulphatase activity of takadiastase. *A*, potassium 4-hydroxy-2-nitrophenylsulphate (I) as substrate, where no inhibition by excess substrate up to 0.005 M is seen; and *B*, potassium 2-hydroxy-5-nitrophenylsulphate (III) as substrate, showing inhibition by excess substrate.

The effect of pH and of substrate concentration on the hydrolysis of I and III by takadiastase is shown in Table 2. In the case of III there was an inhibition by excess of substrate which is shown graphically in Fig. 2.

In optimum conditions with I and III extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4 %.

In the case of the 4-hydroxy-3-nitrophenylsulphate (II) incubation up to 4 hr. only released 6 μ g. of nitroquinol, so that takadiastase appeared to have practically no hydrolytic action towards this ethereal sulphate. This sulphate was also more resistant to acid hydrolysis than its isomer (I), for I is completely hydrolysed by 0.25 N- H_2SO_4 at 98° in less than 10 min., whereas II requires at least 20 min. for complete hydrolysis under the same conditions.

Inhibition of arylsulphatase by 4-hydroxy-3-nitrophenylsulphate

Takadiastase shows very little hydrolytic activity towards potassium 4-hydroxy-3-nitrophenylsulphate (II). This could be due to the inability of the enzyme to combine with II to form the enzyme-substrate complex, or to the forma-

tion of a stable enzyme-substrate compound. If the second condition is correct then potassium 4-hydroxy-3-nitrophenylsulphate should behave as an inhibitor of arylsulphatase since it will combine under certain conditions with all the available enzyme and prevent its action on other hydrolysable substrates. The effect of potassium 4-hydroxy-3-nitrophenylsulphate upon the activity of takadiastase towards potassium 2-hydroxy-5-phenylsulphate was therefore studied.

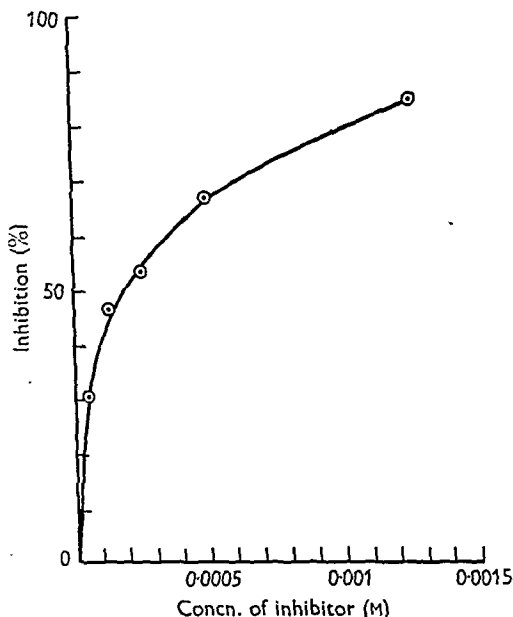


Fig. 3. The inhibition by potassium 4-hydroxy-3-nitrophenylsulphate (II) of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by takadiastase. For conditions, see text.

The hydrolytic action of 0.5 ml. of 2% takadiastase upon 0.0025 M-potassium 2-hydroxy-5-nitrophenylsulphate was measured as above, in the presence of increasing concentrations of potassium 4-hydroxy-3-nitrophenylsulphate. The results (see Fig. 3) showed that the latter substance was highly inhibitory, 50% inhibition being caused by a concentration of about 1.4×10^{-4} M.

The nature of the inhibition. The activity of 2% takadiastase at increasing concentrations of potassium 2-hydroxy-5-nitrophenylsulphate in the absence and presence of 2.5×10^{-4} M-potassium 4-hydroxy-3-nitrophenylsulphate was measured at pH 5.8. The plot of $1/v$ against $1/S$ showed an increase in slope (see Fig. 4) in the presence of the inhibitor, whereas the ordinate intercept had the same value as in the absence of the inhibitor, thus indicating competitive inhibition. According to Lineweaver & Burke (1934) the increase in slope $= K_s I / K_i$, where I is the inhibitor concentration and K_i is the dissociation constant of the enzyme-inhibitor complex. In this particular experiment $K_s (=K_m)$ was 6.8×10^{-4} and K_i was calculated to be 2.4×10^{-6} M. Thus the affinity of potassium 4-hydroxy-3-nitrophenylsulphate for the enzyme was about 300 times ($6.8 \times 10^{-4} / 2.4 \times 10^{-6}$) that of potassium 2-hydroxy-5-nitrophenylsulphate.

*Inhibition of arylsulphatase activity
by some inorganic compounds*

It has been shown that arylsulphatase is inhibited by compounds which react with aldehyde groups, for KCN, K_2SO_3 , phenylhydrazine and NH_2OH are inhibitory (Soda, Egami, Koyama & Horigome, 1939; Rozenfeld & Ruchelman, 1940; Dziaslozynski, 1950). Rozenfeld & Ruchelman (1940) concluded that arylsulphatase contained an aldehyde group in its prosthetic group. Furthermore, it has been reported that oxalate, fluoride, sulphate and phosphate ions and heavy metals inhibit the enzyme (Tanaka, 1938; Inouye, 1929; Dziaslozynski, 1950; Huggins & Smith, 1947). According to Tanaka (1938) and Seligman, Chauncey & Nachlas (1951) $MgCl_2$ activates the enzyme, whereas Hommerberg (1931) claims that this compound inhibits slightly. Other workers have obtained activation of the enzyme by $Fe(OH)_3$, $BaCO_3$ and $CaCO_3$. These various workers, however, were working with arylsulphatases from different sources.

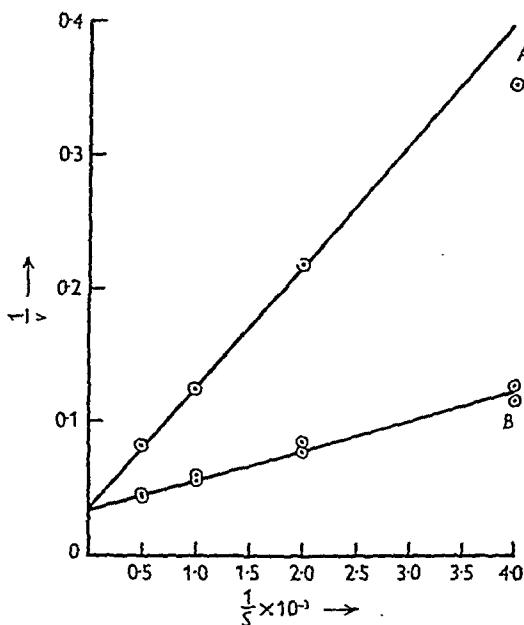


Fig. 4. Competitive inhibition of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by potassium 4-hydroxy-3-nitrophenylsulphate (II). A, in the presence of the inhibitor; B, in its absence (see text). v = rate of hydrolysis, expressed as $\mu g.$ 4-nitrocatechol/hr. S = substrate concn. (M).

Using potassium *p*-chloro- and *p*-nitro-phenylsulphates and takadiastase we have examined the action of a number of compounds on arylsulphatase.

Methods

(a) *Using p-chlorophenylsulphate.* The method described earlier (p. 203) was used, but the 0.4 ml. of substrate solution was replaced by 0.2 ml. inhibitor and 0.2 ml. substrate dissolved in 0.5M-acetate buffer of pH 6.15. The final concentration of substrate was 0.0125M. A similar change was made in the control tubes where 0.2 ml. each of inhibitor and substrate replaced the 0.4 ml. of substrate solution.

(b) *Using p-nitrophenylsulphate.* The inhibitor solutions were made up in M-acetate buffer and adjusted to pH 6.0

with acetic acid or 0.1N-NaOH as necessary. The inhibitor solution (2 ml.), 0.01M-substrate solution (2.5 ml.) and 2% takadiastase (0.5 ml.) were mixed in duplicate and incubated for 1.5 hr. at 37°. The control tubes were incubated in the same way using inhibitor and buffer solutions but omitting the enzyme solution until the end of the reaction. The tubes were then all treated with 0.5 ml. of 2N-NaOH and the liberated *p*-nitrophenol measured.

Table 3. *Effect of inhibitors on arylsulphatase activity of takadiastase*

Compound tested	Final concentration of inhibitor (M)	<i>p</i> -Chlorophenol or <i>p</i> -nitrophenol liberated ($\mu g.$)	Inhibition (%)
<i>p</i> -Chlorophenylsulphate as substrate			
None	—	34.4	—
NaCl	0.02	34.7	0
Na_2SO_4	0.02	34.6	0
$MgCl_2$	0.02	34.5	0
Na_2HPO_4	0.02	27.9	19
Na_2SO_3	0.01	11.0	68
<i>p</i> -Nitrophenylsulphate as substrate			
None	—	20.0	—
KCl	0.02	20.0	0
K_2SO_4	0.02	20.0	0
$MgCl_2$	0.02	14.6	27
Na_2HPO_4	0.02	19.2	4
K_2SO_3	0.02	4.95	75
K_2SO_5	0.002	16.0	20
C_6H_5OH	0.02	18.0	10
NH_2OH, HCl	0.02	2.2	89
NH_2OH, HCl	0.002	7.7	61.5
KCN	0.02	0	100
KCN	0.002	10.7	46.5

Some of our results are given in Table 3. They confirm that compounds reacting with aldehyde groups (KCN, K_2SO_3 , Na_2SO_3 and NH_2OH) inhibit the enzyme. The effect of phosphates was slight but nevertheless evident. $MgCl_2$ (cf. Hommerberg, 1931) seemed to inhibit slightly when *p*-nitrophenylsulphate was used as substrate but not when *p*-chlorophenylsulphate was used.

DISCUSSION

The present work suggests that arylsulphatase can be estimated with almost any phenolic ether sulphate hydrolysed by sulphatase at a reasonable velocity provided that the anion of the liberated phenol absorbs light at a different wavelength from the ethereal sulphate. This probably occurs with most phenols. For the most sensitive methods however, the extinction of the phenolic anion at its wavelength of maximum absorption must be large and of the phenols studied here the *p*-nitro-, *p*-chloro- and *p*-aldehyde-phenols and 4-nitrocatechol seem to satisfy these criteria (see Table 1). The use of *p*-nitrophenol and its sulphate for the assay of sulphatase has already been described (Huggins & Smith, 1947). The convenient maximum absorptions of the *p*-chloro- and *p*-aldehyde-phenol anions

occur in the ultraviolet and are therefore measured with a spectrophotometer covering the ultraviolet. With *p*-nitrophenol, both a colorimeter and a spectrophotometer can be used for measuring the yellow colour of its anion, the latter instrument being more convenient. Since 4-nitrocatechol gives a red colour in alkali, it is readily measured in a colorimeter. We have found the method using 4-nitrocatechol sulphate as substrate readily applicable to the assay of arylsulphatase in animal tissues, the colour being measured with a Spekker absorptiometer. This method is being further developed.

Takadiastase has been shown to hydrolyse the ethereal sulphates of phenol (Neuberg & Kurono, 1923), *p*-cresol (Neuberg & Lindhardt, 1923) *m*- and *p*-chloro-, *p*-bromo-, *o*-, *m*- and *p*-nitro-phenols, quinol, carvacrol, vanillin, *p*-hydroxybenzoic acid, indoxyl, 2-hydroxyquinoline (Neuberg & Wagner, 1925), salicylic acid (Morimoto, 1937), β -naphthol (Noguchi, 1924), oestrogens (Cohen & Bates, 1949), (\pm)-3-methylcyclohexylphenol (Weinmann, 1929) and (+)-*p*-sec-butylphenol (Fromageot, 1929). It

and V, to give I and III respectively, causes the optimum pH for sulphatase activity to become more acid (see Table 4). Thus the optimum for IV is pH 6.3 moving to 6.0 in I, whilst the optimum for V is 6.3 moving to 5.9 in III. There is also in both cases a similar change in optimum substrate concentration which is 0.006M for IV and V and about 0.002M for I and III. Furthermore, K_m for IV and V is about 2.5 times the K_m for I and III ($K_{mIV}/K_{mI}=2.7$ and $K_{mV}/K_{mIII}=2.4$). Thus the introduction of a hydroxyl group into these sulphates increases the affinity of the enzyme for the substrate about 2.5 times.

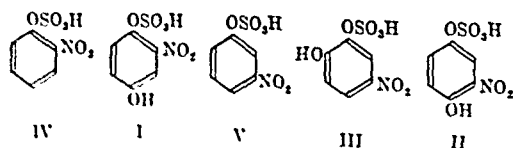
On these grounds it seemed probable that the lack of hydrolytic activity of takadiastase towards II might be due to the formation of a stable enzyme-substrate complex. That this was actually the case was proved by the fact that II competitively inhibited the action of sulphatase on III. It was calculated that the affinity of the enzyme for II was about 280 times that for III and about 50 times that for I.

Table 4. Action of the arylsulphatase of takadiastase upon various ethereal sulphates

Ethereal sulphate	$K_m \times 10^4 M$	Optimum pH	Optimum substrate concentration (M)	Phenol liberated/hr. by 1 g. of takadiastase		1 mg. of the N of the takadiastase preparation ($\mu g.$)
				($\mu g.$)	($\mu mole$)	
<i>o</i> -Nitrophenyl	3.4	6.0-6.5	0.006	1100	7.8	275
<i>m</i> -Nitrophenyl	8.35	5.9-6.5	0.006	1750	12.5	437
<i>p</i> -Nitrophenyl	1.7	6.2	0.015	1272	9.15	318
4-Hydroxy-2-nitrophenyl	1.25	5.9-6.1	0.002	1426	9.2	356
2-Hydroxy-5-nitrophenyl	3.5	5.9	0.0025	837	5.4	209
4-Hydroxy-3-nitrophenyl	(0.024)*	—	—	0	0	—
<i>p</i> -Chlorophenyl	9.7	6.15	0.01	1610	12.5	402
<i>p</i> -Aldehydophenyl	2.1	5.5-6.1	0.0027	960	7.6	240

* K_I measured at pH 5.8.

failed to hydrolyse the ethereal sulphate of (–)-*p*-sec-butylphenol (Fromageot, 1929). Our present results cover eight ethereal sulphates of which *o*-, *m*- and *p*-nitro- and *p*-chloro-phenylsulphates have been studied before. Amongst our results we have two examples of the effect on sulphatase activity of the introduction of a hydroxyl group into the aromatic ring of the nitrophenylsulphates. Thus we can compare 4-hydroxy-2-nitrophenylsulphate (I)



with *o*-nitrophenylsulphate (IV) and 2-hydroxy-5-nitrophenylsulphate (III) with *m*-nitrophenylsulphate (V). The introduction of an OH into IV

Our results also enable us to compare the action of sulphatase on three position isomers, namely the *o*-, *m*- and *p*-nitrophenylsulphates. The affinity ($1/K_m$) of the enzyme is greatest for the *p*- and least for the *m*-isomer, the ratio $1/K_{m,p} : 1/K_{m,o} : 1/K_{m,m}$ being 5:2.5:1.

Although $1/K_m$ is least for *m*-nitrophenylsulphate, more *m*-nitrophenol is liberated per hour by the enzyme from *m*-nitrophenylsulphate than *o*- and *p*-nitrophenols from their corresponding sulphates (Table 4).

The commercial preparation of takadiastase used in this work contained much added lactose and only a small amount of it was mould material. The nitrogen content was 0.4% and the protein content 2.5% (0.4×6.25). On the basis of its nitrogen content and the fact that most of the material of the preparation was added lactose, the original dried mould preparation must be a relatively good source of aryl-

sulphatase, for the enzyme in 1 g. of the takadiastase nitrogen would release 32 mg. of *p*-nitrophenol (see Table 4), for example, from the corresponding ethereal sulphate in 1 hr. at 37°.

Our experiments were carried out at 37°, and Abbott (1947) has reported that the optimum temperature of the enzyme is 50°. From Abbott's results arylsulphatase appeared to be about 50% more active at 50° than at 37°.

SUMMARY

1. A study has been made of the action of the arylsulphatase activity of takadiastase towards the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids.

2. The liberated phenols were determined spectrophotometrically or colorimetrically as their

anions, which have different spectra from the corresponding ethereal sulphates. The spectra of these sulphates are recorded.

3. Optimum pH's, Michaelis constants and optimum substrate concentrations have been determined for these ethereal sulphates.

4. Potassium 4-hydroxy-3-nitrophenylsulphate was not hydrolysed by takadiastase and inhibited the action of the enzyme towards other ethereal sulphates. This inhibition is competitive and is due to the formation of a stable enzyme-substrate complex.

5. A study has also been made of the inhibition of arylsulphatase by inorganic compounds and aldehyde reagents. It is confirmed that arylsulphatase is inhibited by compounds reacting with aldehydes.

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Seasonal Changes in the Carbohydrates of the Jerusalem Artichoke Tuber

By J. S. D. BACON AND R. LOXLEY

Department of Biochemistry, University of Sheffield

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Several workers (Dubrunfaut, 1867; Colin, 1919; Thaysen, Bakes & Green, 1929; Dedonder, 1951c) have noted the change in carbohydrate composition which takes place when the tubers of the Jerusalem artichoke (*Helianthus tuberosus* L.) are stored over the winter. This change may be inferred from the optical rotation of the expressed juice, which changes in the positive direction, and is confirmed

by the observations that in the spring less inulin may be obtained from the tubers and more of the carbohydrate is fermentable. Similar observations have been made on chicory, *Cichorium Intybus* L., by Wolff & Geslin (1918).

Bacon & Edelman (1951) suggested that all these changes could be the result of a redistribution of fructose residues among the many carbohydrate

components revealed by paper-partition chromatography of extracts; a relative increase in the proportion of those of lower molecular weight (the smallest of which is sucrose) should give the mixture a more positive rotation (cf. the rotation of these substances: Dedonder, 1951a) and the increased fermentability could be due to the more rapid action of yeast invertase on them (Dedonder, 1951b).

The following is an account of experiments designed primarily to test this hypothesis. The results also give some indication of the nature of the metabolic changes that take place in hibernating tubers.

METHODS

In order to reduce the actions of enzymes to a minimum the following extraction procedure was adopted, differing in some respects from that described earlier (Bacon & Edelman, 1951):

About 200 ml. of water were brought to the boil in a weighed 500 ml. beaker and kept at the boil while a known weight of tubers (about 100 g.) was added as thin slices cut with a stainless-steel knife. Boiling was continued for a further 3 min., the whole cooled to room temperature, and weighed. The contents of the beaker (usually weighing about 250 g.) were then tipped into a Waring blender and disintegrated without further additions. Samples (about 5 ml.) of this mash were weighed and dried for 24 hr. to determine their content of dry matter.

The mash was then squeezed through madapolam, leaving a small residue, and 50 ml. samples of the filtrate were treated with 5 ml. 30% (w/v) lead acetate ($\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$). The precipitate was filtered off by gravity on a Whatman no. 1 filter paper and to 32 ml. of the filtrate were added 5 ml. 3% (w/v) sodium oxalate. If the supernatant fluid showed a precipitate with one more drop of sodium oxalate solution, measured amounts were added until precipitation of lead was complete.

The optical rotation of the resulting filtrate was read in a 2 dm. tube, and samples were taken for estimation of reducing substance (RS) and total RS (Edelman & Bacon, 1951a). The remaining filtrate was stored over chloroform in the refrigerator. The samples prepared during summer and autumn slowly deposited a precipitate which was presumably inulin; it dissolved when the sample was heated to 80°. Estimations were sometimes repeated on filtrates which had been stored for as long as 9 months and then heated in this way; they showed that no significant changes in composition had taken place.

For chromatographic analysis the filtrate was applied to sheets of Whatman no. 1 paper. These also were stored for several months without detectable changes in the results of the subsequent development and analysis, which were carried out essentially as described by Bacon & Edelman (1951). The procedure was modified in certain details: (1) the spots for analysis were applied in groups of four, between guide spots, in such a way that all the material for analysis was delivered consecutively from the micrometer syringe, thereby decreasing considerably the error of this measurement; (2) the 'paper blanks' were usually cut from a position below free fructose, and deductions made from the ketose content of particular spots in proportion to the

area of paper they occupied. The recovery of ketose from the chromatograms was in most analyses between 95 and 100%, and never less than 90%.

MATERIALS

The artichokes used were those grown during 1949, and described by Bacon & Edelman (1951). Small tubers of uniform size and regular shape were planted 1.5 ft. (50 cm.) apart in the same plot in the western outskirts of Sheffield, about 550 ft. (170 m.) above sea level. These were the source of most of the material analysed. A few tubers of similar size and shape, lifted at the same time, were planted at Telham, about 2 miles (3 km.) south-east of Battle, Sussex, at an elevation of about 400 ft. (120 m.). In both situations the plants made considerable vegetative growth, but only in Sussex were flowers produced.

At intervals of about 2 weeks a whole plant was lifted, the tubers taken to the laboratory as quickly as possible, and extracted within an hour. All the tubers were given a preliminary washing under the cold tap, dried with filter paper and weighed. Two 100 g. samples of whole tubers were taken, and washed more thoroughly before extraction. The yield of tubers from single plants varied considerably not only in number and weight but also in shape. No systematic attempt was made to choose representative samples from each plant.

The tubers were not removed from the ground in the autumn; they began to grow again in 1951 rather later in the spring than in 1950.

RESULTS

Calculations from analyses. For the samples taken in August and September the dry weight of the tubers was determined directly by drying slices, but in October and afterwards it was calculated from the dry weight of the mash, assuming no losses of dry matter during the boiling, and complete mixing of the added water with the pulp. For some samples both methods were used.

The free and total RS/100 g. fresh weight of tuber were calculated from the analyses of the madapolam filtrate, neglecting the small residue left on the cloth. It was assumed that the additions of lead acetate and sodium oxalate solutions diluted the resulting filtrates to a volume equal to the sum of the volumes mixed.

The ketose as percentage total ketose recovered of each spot in the paper chromatogram cannot be assumed to represent exactly the distribution of fructose in the various components, nor can the absolute amounts of most of these components be calculated, since, with the exceptions of fructose and sucrose, the pure substances were not available.

Changes in composition with age

Tables 1 and 2 summarize the results of analyses on tubers from 20 July 1950, when they were almost indistinguishable from roots, until May 1951, when shoots 25 cm. long had developed on some of them.

Table 1. *Dry matter of tubers and total reducing substances, ketose and optical rotation of tuber extracts*

(Figures marked * are for a single extraction, all other figures are for duplicate extractions. All extracts were made on the date recorded except for those marked † which were made (30 September 1950) 2 days, (4 December 1950) 7 days, (14 April 1951) 3 days, and (9 June 1950) 3 days later, respectively. ‡ tubers from Sussex.)

Date dug up	Dry matter (g./100 g. fresh tuber)	Total RS (g./100 g. fresh tuber)	$[\alpha]_D$ calculated on total RS	Ketose as % total RS
20 July 1950	12.4*	4.7*	-10.4*	85*
22 Aug. 1950	15.5	11.4	-22.5	95
28 Aug. 1950	16.2	10.0	-20.1	98
4 Sept. 1950	15.6	11.0	-21.0	96
11 Sept. 1950	15.9	11.6	-22.4	95
18 Sept. 1950	17.0	13.3	-21.1	—
25 Sept. 1950	16.8	11.8	-19.8	94*
30 Sept. 1950†‡	13.9	9.9	-23.3	97*
3 Oct. 1950	18.4	12.7	-19.4	—
9 Oct. 1950	18.9	14.3	-17.5	—
16 Oct. 1950	18.1	13.7	-16.1	—
23 Oct. 1950	19.4	14.1	-16.3	93*
30 Oct. 1950	20.4	14.8	-16.0	94
6 Nov. 1950	20.7*	15.3*	-9.5	89
9 Nov. 1950	21.5	16.0	-7.5	90*
14 Nov. 1950	20.8	14.5	-4.0	86
20 Nov. 1950	20.8	15.5	-4.9	—
26 Nov. 1950	21.0	15.3	-1.8	—
4 Dec. 1950	20.8	14.0	-1.2	90*
4 Dec. 1950†	33.5	23.9	-3.0	—
19 Dec. 1950	21.1	14.2	+2.0	88
2 Jan. 1951	21.5	15.2	+3.0	—
17 Jan. 1951	20.0	14.0	+5.4	79*
12 Feb. 1951	19.9	13.6	+4.1	—
5 Mar. 1951	20.1	15.6	+0.5	—
2 Apr. 1951	22.0	13.7	+3.6	84
14 Apr. 1951†‡	22.1	16.8	+8.1	77
25 May 1951	15.2	10.9	-16.6	88*
9 June 1950†	10.8	6.3	-9.2	81

Table 2. *Distribution of ketose among the components of tuber carbohydrate*

(Results refer to the same extracts as those in Table 1.)

Date dug up	$[\alpha]_D$ calculated on total RS	Ketose as % of ketose recovered from chromatogram					Recovery of ketose (%)
		Spot F	Spot 1	Spot 2	Spot 3	Spot 4	
30 July 1950	-10.4	—	4.3	5.1	6.1	—	102
22 Aug. 1950	-22.4	—	1.8	1.8	2.5	3.0	98
22 Aug. 1950	-22.6	—	1.8	1.8	2.5	3.0	102
28 Aug. 1950	-18.8	—	2.0	2.2	2.8	3.1	101
28 Aug. 1950	-21.4	—	2.2	2.4	2.8	3.1	99
30 Sept. 1950	-24.9	—	1.4	1.7	2.0	2.7	98
23 Oct. 1950	-16.8	—	2.7	3.0	3.5	4.2	100
9 Nov. 1950	-6.9	—	5.7	4.9	6.2	6.1	95
4 Dec. 1950	-0.7	—	6.8	6.3	8.4	8.2	99
17 Jan. 1951	+6.5	—	10.1	8.0	10.7	9.2	98
2 Apr. 1951	+3.3	—	9.1	7.7	7.2	9.7	95
2 Apr. 1951	+4.0	—	9.5	8.0	10.7	10.2	93
14 Apr. 1951	+7.7	1.8	13.7	10.9	12.3	11.2	97
25 May 1951	-19.0	9.6	7.9	4.7	5.5	5.6	—
25 May 1951	-14.2	7.5	7.0	4.6	5.8	6.6	96
9 June 1950	-7.4	25.6	18.6	8.7	7.8	6.8	92

(These tables may be compared with Tables 1 and 4, Bacon & Edelman, 1951.) To them have been added analyses of some old tubers still present in actively growing plants during June 1950; these tubers were still turgid, and conform to the trends in composition which were evident during May of the following year.

The dry weight as percentage of fresh weight increased during the period of sub-aerial growth, but thereafter remained almost constant until the tubers began to sprout, when it declined sharply. There was a considerable increase in the total weight of tuber per plant; in July the total weight was 14 g., in August about 200 g., and in September,

October and November never less than 300 g. and often 600 g. or more. The increase was due partly to increase in the number of tubers, but more to increase in their size. In all plants there was very considerable disparity in size between tubers, the largest ones weighing more than 100 g., the smallest 5 g. or less. Despite this variation the dry weights of duplicate samples agreed to within 10 % in practically all cases.

Total RS as a percentage of the fresh weight of the tubers also showed an increase until the end of October when it remained more or less steady until growth began in April. It is not possible with the data available to estimate the changes in total carbohydrate taking place in a single tuber between November and April, but in view of the small changes in dry weight and total RS as proportions of the fresh weight of tuber it is reasonable to suppose that this change also must have been small and could not exceed 10 %.

Free RS was very small during the period from July to April, constituting 1 % or less of the total RS. Larger amounts found in the Sussex tubers in April (3.8 % of total RS) and in the Yorkshire tubers in May 1951 (10.9 %), and June 1950 (27.8 %), were mainly accounted for as free fructose (see Table 2); there was no appreciable free glucose in any of the extracts examined.

Ketose as percentage of total RS decreased noticeably, displaying differences similar to those recorded by Bacon & Edelman (1951; Table 4); in Yorkshire from 95 to 96 % in August it decreased to 85 % in March. The method of estimation used for ketose is not as satisfactory as that for reducing substances, but even if allowance is made for this, the decrease implies an increase in non-ketose RS (glucose) of about three- to four-fold.

This increase in the proportion of glucose was accompanied by an increase in components with greater R_f values. As already noted by Bacon & Edelman (1951), the fructose was distributed fairly evenly among these components; in particular there was no marked change in spot 1 relative to spots 2-4. The sum of the amounts of ketose in spots 1-4 as a percentage of the total ketose estimated, which may be taken as a measure of the proportion of components of lower molecular weight, increased in Yorkshire from only 9 % in August to 38 % in April, and in Sussex from 8 % in September to 48 % in April, the greatest change taking place in the 2 months following the death of the foliage in the last week of October.

Parallel with this increase in the proportion of components of lower molecular weight there took place a change of optical rotation in the positive direction (Fig. 1; a line has been drawn to emphasize the consistent trend shown during October, November and December, despite the fact that

analyses were made on individual plants). In August the rotation, expressed as an $[\alpha]_D$ on the basis of the total reducing substances, was -22.5° ; by April it had risen to $+3.5$. Again, the most rapid

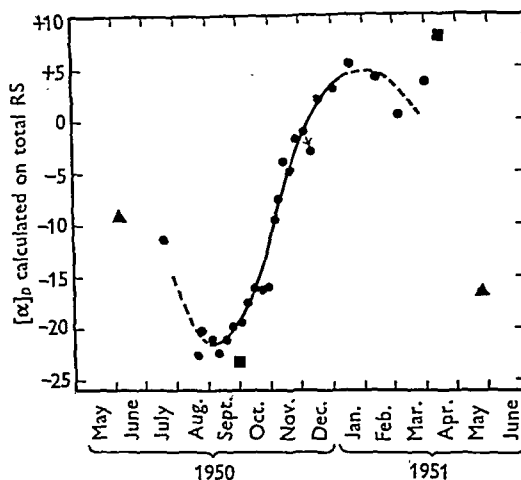


Fig. 1. Seasonal changes in the optical rotation of extracts, plotted from the data in Table 1. Yorkshire-grown tubers: ●, free RS small; ▲, free RS large. Sussex-grown tubers, —, —.

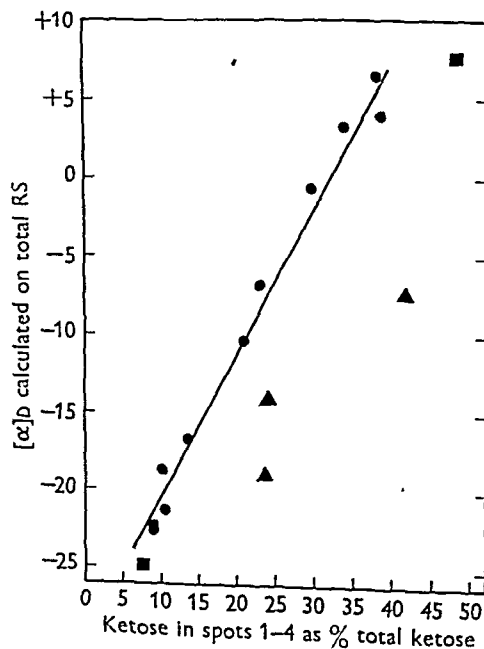


Fig. 2. Relation between the optical rotation of extracts and the sum of the ketose contents of spots 1-4 as percentage total ketose recovered, plotted from the data in Table 2. Yorkshire-grown tubers: ●, free RS small; ▲, free RS large. Sussex-grown tubers, —, —.

rate of change took place following the death of the foliage. The close connexion between rotation and distribution of ketose in spots 1 to n is shown by Fig. 2, in which the ketose in spots 1-4 as a percent-

age of the total ketose estimated is plotted against the $[\alpha]_D$ calculated from the total RS; all the points for extracts containing little free RS lie on or near a straight line.

The single extract made in July 1950 resembled the extracts obtained in November as far as $[\alpha]_D$ and the ketose distribution were concerned. It differed from them in its low content of total RS.

That the changes observed are not simply the result of the rise in temperature following removal from cold ground was shown by analyses of tubers dug up in December and kept at laboratory temperature for a week (Table 1, 4 December 1950). Although the tubers lost considerable amounts of water the $[\alpha]_D$'s of the extracts were not significantly different from those of tubers of the same plant extracted immediately after removal from the ground.

DISCUSSION

It has been shown by Bacon & Edelman (1951) and by Dedonder (1950) that the carbohydrates of the artichoke tuber are more numerous than had been thought previously, and both have suggested that they constitute a series of non-reducing substances which may be considered as being formed from sucrose by the addition of one or more fructofuranose residues.

The results presented here show that in the living plant the relative amounts of these substances change in a consistent way during growth, dormancy, and germination of the tubers. This change is accompanied by a change in the optical rotation of the deproteinized extract which has a linear relationship to it, thus strengthening the idea that each 'spot' on the paper chromatogram represents a single substance. It is significant that the single extract made in July 1950 conforms to the relationship established with later samples, because although it has a low total RS content (as would be expected from the results for August and September), its $[\alpha]_D$ and distribution of ketose resemble those of November samples.

Dedonder (1951*b*) has described the action of yeast invertase on spots 1-4 (*A*, *B*, *C*, *D* in his terminology); from this it might be expected that the juice from tubers harvested in the spring would be fermented more readily by yeast, as has been observed before (Thaysen *et al.* 1929).

Certain suggestions as to the nature of the metabolic changes occurring in the tubers may be made on the basis of the present results. The most striking change is the increase in relative glucose content, shown by estimation of ketose as percentage of total RS, and also by the increase in the proportion of the spots 1-4, which have relatively high glucose contents. Such a change could be

achieved by the action of the hydrolytic enzyme found in extracts (Edelman & Bacon, 1951*a*) followed by utilization of the fructose produced, but this would involve the disappearance of about three-quarters of the fructose present in October. Such an explanation would seem to be excluded by the indirect evidence available. Colin (1919) rejected the idea that the inulase of Green (1888) was responsible for the changes that he observed.

An alternative explanation is that a slow conversion of fructose to glucose takes place (*cf.* Dedonder, 1951*d*), probably not involving the formation of free sugars, and that from this glucose more sucrose is produced. Then by the transfructosidation reaction described by Edelman & Bacon (1951*b*) a redistribution of fructose residues occurs, increasing the amounts of the substances of smaller molecular size at the expense of those of large.

This would imply that during the period of carbohydrate storage the tendency is for substances of high molecular weight (and hence of low glucose content) to be formed. The analysis of the July tubers possibly represents the earliest stage of storage, when the amount of polysaccharide laid down is still small.

The large amount of free fructose found in sprouting tubers could perhaps be due to a very greatly increased activity of the hydrolytic enzyme system during the extraction procedure, but more probably indicates the manner in which carbohydrate is mobilized. (In unpublished experiments we found no increase in the units of hydrolytic enzyme extractable from stored tubers during the period 8 March to 9 May 1949.) The decrease in proportion of spots 1-4 probably indicates that glucose is also being liberated, perhaps by hydrolysis of sucrose.

It is not easy to see why the composition of the tubers should change in this way; it may represent a reversal of the process by which polysaccharide is laid down during the period of photosynthesis. Similar changes would presumably take place if the tubers were harvested and stored in a suitably cool and not too dry place.

SUMMARY

1. Analyses of dry matter, and of the ketose and combined reducing substances in aqueous extracts, have been made on tubers of the Jerusalem artichoke during the period June 1950 to May 1951, confirming the occurrence of a change in carbohydrate composition during the winter months, already noted by several investigators.

2. A change in the positive direction of the optical rotation of extracts has been correlated with an increase in the proportion of carbohydrate com-

ponents of lower molecular weight estimated by quantitative paper-partition chromatography.

3. The significance of these findings is discussed in relation to the structures proposed by Dedonder (1951a) and Bacon & Edelman (1951) for the tuber

carbohydrates and a suggestion is made as to the nature of their metabolic interconversion.

We are very grateful to Dr R. Dedonder for sending us a copy of his Thesis on 'Les glucides du topinambour', which contains much experimental detail not yet published.

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Studies in Carotenogenesis

4. NITROGEN METABOLISM AND CAROTENE SYNTHESIS IN *PHYCOMYCES BLAKESLEEANUS*

By T. W. GOODWIN AND J. S. WILLMER

Department of Biochemistry, The University of Liverpool

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Preliminary experiments on the relationship between nitrogen metabolism and carotenogenesis in *Phycomyces blakesleeanus* (Garton, Goodwin & Lijinsky, 1951) suggested that carotene was not synthesized when well formed mycelial mats were transferred to a medium containing glucose but no assimilable nitrogen. The present paper records a full investigation into this aspect of the problem of carotene formation in *Phycomyces*.

Part of this work has already been briefly described (Goodwin, Lijinsky & Willmer, 1951).

EXPERIMENTAL

The general cultural conditions, the technique of mycelial transfer, and the analytical methods for dry weight, lipid, and carotene determinations have previously been described in detail (Garton *et al.* 1951; Goodwin & Lijinsky, 1951). Nitrogen determinations were carried out using the micro-Kjeldhal method of Markham (1942).

RESULTS

Carotene production and nitrogen assimilation in growing cultures

Mycelia of *Phycomyces* cultured on media containing 0.2% (w/v) of L-asparagine and either 2.5, 1.5 or 1.0% (w/v) of glucose (unless otherwise stated the salt and aneurin concentrations are those used by Garton *et al.* 1951) were harvested at various times after inoculation and analysed for dry weight,

lipid, carotene and nitrogen. The results obtained using the medium containing 2.5% glucose are recorded in Fig. 1. From this figure it will be seen

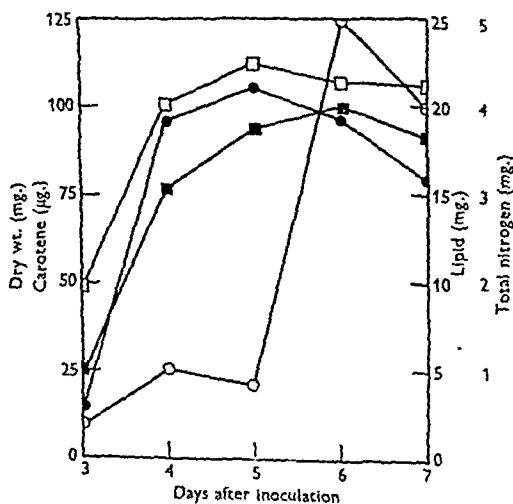


Fig. 1. The dry weight, lipid, carotene and nitrogen content of developing *Phycomyces* cultured in an 8 oz. medicine bottle on 15 ml. of a standard medium (Garton *et al.* 1951) containing 2.5% (w/v) glucose and 0.2% (w/v) asparagine. □—□, dry weight; ○—○, carotene; ■—■, lipid; ●—●, nitrogen.

that, assessed by cessation of either dry weight production or nitrogen assimilation, growth must be complete before carotene synthesis proceeds at all

rapidly. Growth is complete within 4 or 5 days, but at this time carotene formation is only about 16% of the final amount, which is rapidly synthesized within the next 2-3 days. Very similar results, which are not recorded here, were obtained using the medium containing 1.5% (w/v) of glucose, except that the amount of carotene synthesized was not as great. As would be expected from the previous results of Garton *et al.* (1951), after growth had ceased no further carotene was produced on the medium containing 1% of glucose owing to the absence of dissimilable glucose.

considerable amount of the nitrogen is lost to the medium. Because dry weight production is directly proportional to, whilst the nitrogen uptake is independent of, the glucose concentration of the medium, young mycelia growing on a glucose-poor medium have a very high nitrogen concentration. This can be seen from Table 1 which also demonstrates that owing to the different rates at which nitrogen is lost from the mycelia, its concentration in all mycelia tends to be the same after about 7 days' growth. In older cultures, the nitrogen concentration of the mycelia grown on the glucose-rich medium again tends to be lower; this is due to the maintenance of the mycelial weight in old cultures when sufficient glucose is present. On the glucose-poor media old cultures tend to decrease in dry weight.

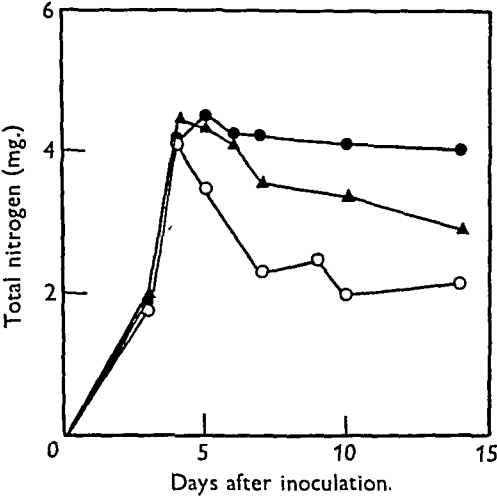


Fig. 2. The nitrogen content of developing *Phycomyces* cultured on media containing 0.2% (w/v) of L-asparagine but varying amounts of glucose. ○—○, 1% glucose; ▲—▲, 1.25% glucose; ●—●, 2% glucose.

Table 1. Total nitrogen concentration in mycelia of *Phycomyces* grown on media containing varying amounts of glucose

Age of culture (days)	Nitrogen concentration (%) in medium containing		
	1% (w/v) glucose	1.5% (w/v) glucose	2.5% (w/v) glucose
3	6.8	8.7	7.9
4	12.3	6.1	5.4
5	5.17	4.3	4.5
6	5.8	5.3	4.3
7	4.3	4.8	4.6
10	4.9	5.0	3.7
14	5.2	4.8	4.0

The results obtained for the nitrogen uptake on the different media are worthy of separate consideration. Fig. 2 shows that the maximal nitrogen content occurs 3-4 days after inoculation, and is the same irrespective of the glucose concentration of the medium. In the case of mycelia grown on media containing excess glucose (2.5%, w/v) most of this nitrogen is retained in the mycelia as they age. If, however, the media contain insufficient glucose it is clear from Fig. 2 that, as the mycelia mature, a

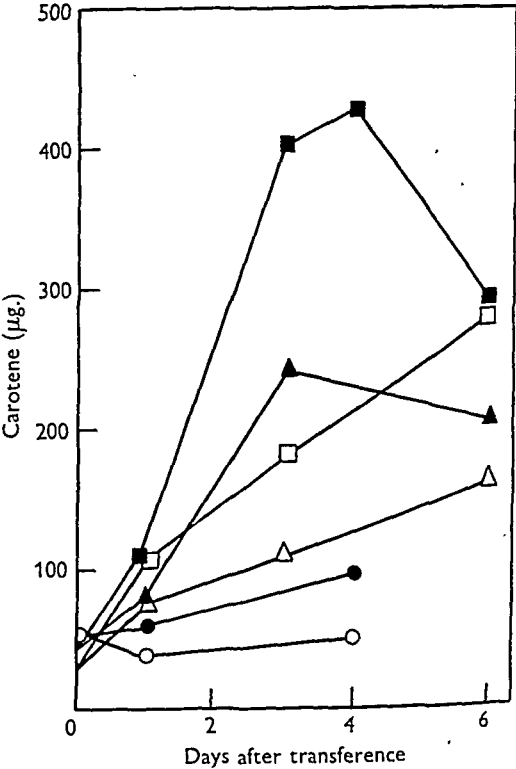


Fig. 3. The carotene content of mycelia after transference, when 4 days old, to hard media containing 2.5% glucose and either: ■—■, 0.2% (w/v) L-asparagine (unbuffered medium); ▲—▲, no nitrogen (unbuffered medium); ●—●, 0.2% (w/v) L-asparagine (buffered medium, pH 7); ○—○, no nitrogen (buffered medium, pH 7); □—□, 0.2% (w/v) L-asparagine (medium with high salt concn. see Table 2); △—△, no nitrogen (medium with high salt concn.).

Carotene synthesis in the presence and absence of assimilable nitrogen

The results just described suggest that the failure of Garton *et al.* (1951) to observe carotene synthesis by well developed mycelial mats dissimilating

glucose in the absence of nitrogen was due to a factor other than the absence of nitrogen from the medium. The only cultural difference in the transference experiments of Garton *et al.* (1951) compared with the other experiments described by them was that the formed mycelia were transferred to the standard medium buffered at pH 7.0; all other media were unbuffered. A series of experiments was thus undertaken to test whether the buffering of the medium inhibited carotenogenesis under conditions known to be normally favourable to synthesis. As the buffering of the medium necessitated a considerable increase in salt concentration of the medium, the effect of increasing this, irrespective of buffering power, was also investigated. The concentrations of the salts used to prepare the various media are recorded in Table 2 and the experimental results in

Fig. 3 and Table 3. Fig. 3 shows that carotene synthesis on an unbuffered medium with a normal salt concentration is considerable, whether the medium contains nitrogen or not. On buffered media, on the other hand, no carotene is synthesized on the nitrogen-free media (there is actually a slight loss of carotene during the first day after transference), whilst only a very small amount is synthesized when nitrogen is present. On media with only slight buffering capacity but with twice the salt concentration of the buffered media, i.e. ten times the normal salt concentration, carotenogenesis still takes place, but to a somewhat lesser degree compared with the standard medium. Table 3 shows that dry weight production is not appreciably affected by the ionic condition of the medium; lipogenesis, on the other hand, is stimu-

Table 2. Salt concentration in media used to investigate the effect of buffering and salt concentration on carotene synthesis

Constituent	Concentration of salt % (w/v)				
	Standard medium	Medium buffered to pH 7.0	Unbuffered medium with salt concn. equal to twice that of buffered media	Medium buffered to pH 6.0	Medium buffered to pH 5.2
MgSO ₄ ·7H ₂ O	0.05	0.05	0.5	0.05	0.05
KH ₂ PO ₄	0.15	0.363	1.5	0.817	0.885
Na ₂ HPO ₄	0.00	0.568	0.00	0.094	0.0237

Table 3. Amounts of dry weight, lipid and carotene produced by well formed (4-day-old) mats of *Phycomyces* after transference to buffered and unbuffered media containing 3.0 % (w/v) of glucose and either no nitrogen or 0.2 % (w/v) of L-asparagine as the sole nitrogen source

(The salt concentrations of the various media are given in Table 2. The mats were grown on filter paper in Petri dishes (7.5 cm. diameter) containing 45 ml. of medium and transferred to similar dishes containing the same amount of medium (see Goodwin & Lijinsky, 1951).)

Time after transference (days)	With nitrogen				Without nitrogen			
	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Carotene (p.p.m.)
Standard medium								
1	383	79	20.6	210	113	68	60.2	500
3	387	74	19.1	1060	103	35	34	2070
5	294	45	15.3	1070	84	21	25	2410
Medium buffered at pH 7.0								
1	142	42	29.6	—	34	18	52.9	0
2	331	110	33.2	45	101	41	40.6	0
3	356	122	34.2	73	72	39	54.1	0
4	341	125	36.6	111	—	—	—	0
5	382	98	34.6	74	71	32	45.1	0
Medium with high salt concentration (unbuffered)								
1	237	70	29.5	250	(-17)	(-2)	—	—
3	317	87	27.5	420	—	15	25.4	1110
5	369	112	30.4	630	80	80	37.5	1380

Table 4. *Dry weight and lipid production of Phycomyces cultured on the standard medium (containing 3 % (w/v) glucose) buffered at various pH values*

Age of culture (days)	Medium buffered at pH 7.0			Medium buffered at pH 6.0			Medium buffered at pH 5.2			Unbuffered medium		
	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)	Dry wt. (mg.)	Lipid (mg.)	Lipid (%)
4	57.5	17.4	30.1	48.5	—	25.6	59.1	17.0	28.8	63.5	17.5	27.5
7	103.0	34.7	33.7	109.2	36.4	33.3	98.3	28.5	28.9	83.7	14.3	17.1
11	104.8	33.6	32.0	104.9	26.3	25.0	97.7	21.7	22.1	69.7	10.7	15.3
17	93.4	14.1	15.0	91.8	16.8	18.3	96.5	18.2	19.0	—	—	—

lated on media containing a high salt concentration probably irrespective of pH (see later). Lipogenesis was also stimulated in the absence of nitrogen; up to 50 % of the dry weight formed by a well developed mat, after transference to a medium not containing nitrogen and buffered at pH 7.0, is lipid.

medium after inoculation with spore suspension), whilst those of the other buffered media fell to 4.1 and 3.8 respectively; the final pH of the control (unbuffered) medium was 3.2. Carotene synthesis varied inversely with the pH of the medium. The total dry weight production was not affected by the pH of the medium, whilst, as in the case of transferred cultures, lipogenesis was affected in exactly the opposite way to carotenogenesis, being considerably higher in media of high pH.

DISCUSSION

The results recorded in Fig. 1 show that, in the presence of excess glucose and a source of readily assimilable nitrogen, the major portion of carotene synthesis of *Phycomyces* occurs after growth (dry weight production and/or nitrogen assimilation) is complete. It appears, then, that most of the carotene is synthesized from the products of the dissimilation of glucose, quite probably under anaerobic conditions, for carotene accumulates in the surface of the mycelium in contact with the medium and, as the cultures are static, conditions in this region must be almost completely anaerobic. Carotenogenesis in *Phycomyces* could thus be an example of fermentative assimilation, a type of synthesis known to occur in yeasts (Clifton, 1946).

The question naturally arises as to how the observation of Goodwin & Lijinsky (1951), that, in the presence of suboptimal amounts of glucose, L-valine and L-leucine stimulate carotenogenesis, fits in with these just reported. It will be seen from Fig. 1 that a small amount of carotene is always synthesized as the mycelium is growing, thus indicating that the enzyme systems necessary for this synthesis are present in growing mycelia. If the amino-acid being used as the nitrogen source yields on deamination a carbon residue readily incorporated into the carotene molecule, then obviously the amount of carotene synthesized as the fungus is growing will be increased; this is what occurs with L-valine and L-leucine.

There is now no doubt that the inability of Garton *et al.* (1951) to observe carotenogenesis in *Phycomyces* mats dissimilating glucose in the absence of nitrogen

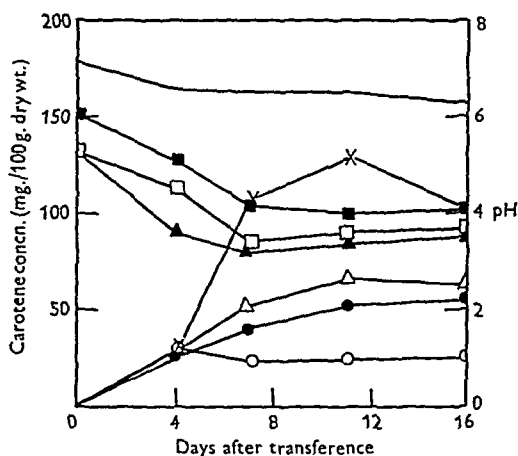


Fig. 4. The concentration of carotene in mycelia grown on standard medium buffered at various pH values; ○—○, medium buffered at pH 7.0; ●—●, medium buffered at pH 6.0; △—△, medium buffered at pH 5.2; ×—×, unbuffered medium.

Also the corresponding changes in the pH of the media during growth; —, medium initially buffered at 7.0; ■—■, medium initially buffered at 6.0; □—□, medium initially buffered at 5.2; ▲—▲, unbuffered medium.

On unbuffered media the synthesis of carotene by developed mats is relatively greater in the absence of nitrogen (Table 3), for the concentration of carotene in the dry matter produced by a mat dissimilating glucose in the absence of nitrogen is about twice that observed when nitrogen is present in the medium.

The results of a series of experiments carried out to see if the effect of buffering the medium, just described for transferred cultures, is observed in ordinary cultures are recorded in Table 4 and Fig. 4. Three buffered media were used at pH 7.0, 6.0 and 5.2 (see Table 2 for the salt concentration). It will be seen from Fig. 4 that the pH of the medium buffered at 7.0 remained almost constant throughout the experiment at about 6.5 (the pH of the

was due to their media being buffered at about pH 7. The present results show that in any medium in which the pH is not allowed to fall to 3.2, carotene synthesis is considerably inhibited. A probable explanation of this is that, assuming that the pigments are produced by fermentative assimilation, the changes in pH alter the pathway of glucose dissimilation, and the production of the building units required for carotene synthesis is reduced. The classic example of this is the very sharp optimal pH for the production of citric acid by *Aspergillus niger*, and the increased production of acids other than citric at other pH values (Bernhauer, 1926). The reduced synthesis by mats transferred to unbuffered media with a high salt concentration (Fig. 3) is probably due to the slight buffering power of this medium rather than to any osmotic effects, for it was noted that during the experiment the pH of these media never fell below 3.8. The pH of normal media falls to 3.2 (Fig. 4).

The present work has demonstrated that lipid production in *Phycomyces* depends also on the pH of the medium, the higher the pH the greater the lipogenesis. Again, this can be explained on the assumption of variation in the products of glucose dissimilation according to the pH of the medium. Similar variations in lipid production have been observed in *A. niger* (Pontillon, 1930) and *A. fisheri* (Prill, Wenck & Peterson, 1935).

Nitrogen metabolism

The observations reported here on the assimilation of nitrogen by *Phycomyces* fall into line with those by Steinberg (1939) on *A. niger*, i.e. the amount of nitrogen removed from the medium is very much the same regardless of the glucose concentration of

the medium. The present work further shows that on media containing only a low concentration of glucose a considerable part of the nitrogen assimilated during the early stages of growth is rapidly lost as the mycelia age, probably owing to the utilization of nitrogen-containing material for the production of energy. On media containing adequate amounts of glucose little or no excretion takes place.

SUMMARY

1. The major portion of the carotene synthesized by *Phycomyces blakesleeanus*, using a readily assimilable source of nitrogen which does not also provide a specific carotene precursor, is produced only after the mycelial mat is fully grown, as measured by dry weight production and/or nitrogen assimilation.

2. Media buffered at high pH values (5.2-7.0) support normal growth of *Phycomyces*, although carotenogenesis is almost completely inhibited. Lipogenesis, on the other hand, is stimulated under these conditions.

3. Well formed mats of *Phycomyces* dissimilating glucose can synthesize relatively more carotene in the absence of assimilable nitrogen than in its presence. Under these conditions lipid synthesis is also relatively greater on the nitrogen-free media.

4. Nitrogen uptake by *Phycomyces* is independent of the glucose concentration of the medium. When the glucose concentration of the medium is low a considerable amount of the assimilated nitrogen is lost as the mycelia age.

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The Estimation of Acetone Bodies

By CHRISTIAN THIN AND A. ROBERTSON

*Department of Hygiene and Preventive Medicine, Royal (Dick) School of Veterinary Studies,
University of Edinburgh*

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In a study of the pathogenesis of bovine ketosis it was felt desirable to be able to follow in some detail the changes in all three ketone bodies (acetone, acetoacetic acid and β -hydroxybutyric acid) which have been shown by various workers to occur in excessive quantities in blood, milk and urine during the course of that disorder. Hitherto, little or no attempt has been made at complete differentiation as both acetone and acetoacetic acid are usually determined in the one fraction. Moreover, *iso*-propanol which we have found (Robertson, Thin & Stirling, 1950) to be present in this disease and in pregnancy toxæmia of ewes, has been unsuspected hitherto in such conditions and, as it is oxidized to acetone by chromic acid, its presence will undoubtedly have influenced the estimation of total ketones by various methods. In this paper the term 'Total Acetone Bodies' is used, therefore, to include acetone, acetoacetic acid, β -hydroxybutyric acid and *iso*-propanol.

The accurate determination of free acetone in the presence of acetoacetic acid is difficult. Van Slyke's method, using Denigès reagent (Van Slyke, 1917, 1929) which has frequently been utilized in ketosis studies, cannot be used to estimate free acetone; in addition, it is not very sensitive as we have found that no precipitate is formed with small amounts of acetone bodies of the order of 5 mg./100 ml. or less. Moreover, using this method, *isopropanol* appears to be oxidized to acetone partly in the acetone plus acetoacetic acid fraction, and partly in the β -hydroxybutyric acid fraction, so making the accurate determination of the individual acetone bodies impossible.

Attempts at using the distillation methods of Shaffer & Marriott (1915) and Behr (1928, 1940) for free acetone by distillation under reduced pressure, or by blowing a fine stream of air through the solution at room temperature, in order to prevent acetoacetic acid decomposing, gave poor results, as with quantities likely to be present in biological fluids, only a small proportion of the acetone could be recovered. The method of Werch (1940, 1941), utilizing diffusion into Nessler's solution in Conway microdiffusion units, though providing a very delicate qualitative test, did not give accurate

quantitative results; the time for the appearance of the precipitates varied and they could not be estimated gravimetrically with any degree of success. The following method was therefore devised, combining with some modification the oxidation technique of Greenberg & Lester (1944), the diffusion method of Werch (1940, 1941) and Seifert (1948), and the colorimetric technique of Behr & Benedict (1926). It depends on the development of an orange to red coloration when an alkaline solution of salicylic aldehyde is left in the presence of acetone, the depth of colour formed being directly proportional to the amount of acetone present. This can be used to estimate free acetone, the acetone formed by hydrolysis of acetoacetic acid, and that formed by chromic acid oxidation of β -hydroxybutyric acid and of *isopropanol*.

METHODS AND RESULTS

Apparatus. Microrefluxing apparatus (Greenberg & Lester, 1944); Conway microdiffusion units.

Reagents. Approx. 0.15N-Ba(OH)₂ and approx. 2.5% (w/v) ZnSO₄·7H₂O (Greenberg & Lester, 1944); 20N-H₂SO₄; 10% (w/v) K₂Cr₂O₇; 1.5% (w/v) K₂Cr₂O₇ in 15.6N-H₂SO₄; 20% (w/v) acetic acid; 4N-KOH; salicylic aldehyde supplied by British Drug Houses Ltd.

Colour reagent. To 1 ml. of salicylic aldehyde are added 8 ml. of 4N-KOH and the solution well mixed; 2 ml. are used for each estimation.

Preparation of standard graphs. A standard solution of acetone (3 ml.), prepared according to Behr & Benedict (1926) and containing 0–10 mg. acetone/100 ml. of solution are placed in the outer chamber of a Conway dish with a few drops of 20% (w/v) acetic acid; 2 ml. of the colour reagent are placed in the inner chamber. The lid is put on firmly, after greasing the rim, and the dish is left 0.5–3 hr. in an incubator at 37°, or any other standard time and temperature that is convenient; alternatively, it may be kept at room temperature overnight, when the maximum colour is obtained regardless of temperature. It should not be left standing, however, more than 20 hr. as the colour reagent tends to blacken after this interval. A blank is run using water instead of the standard solution.

After the requisite time 0.5 ml. of the coloured solution in the central chamber is added to 2 ml. of water in a test tube, and the resulting solution read in the Spekker photoelectric colorimeter against the blank similarly prepared, using 2 ml. cells and a blue-green filter (Ilford 603).

Fig. 1 shows a graph prepared by estimating known amounts of acetone. It will be seen that Beer's Law is strictly adhered to over the range 0-10 mg./100 ml. Table 1 shows that results with standard acetone solutions are reproducible within $\pm 4\%$.

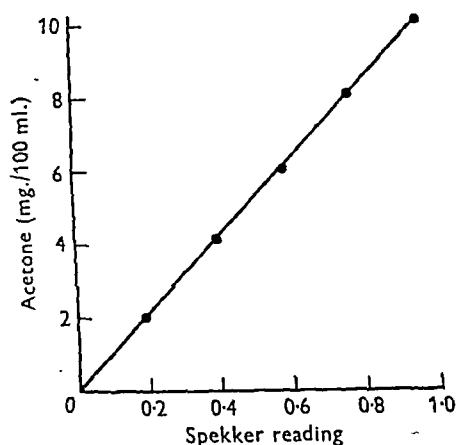


Fig. 1. Standard curve for acetone, measuring the colour produced with alkaline salicylic aldehyde against a blank after standing 2 hr. at 37° . Filter: blue-green, Ilford 603.

Table 1. Reproducibility using standard solutions of acetone

Time interval (hr.)	Theoretical result (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
2	7.92	7.80	-1.5
2	7.92	8.00	+1.0
Overnight	7.92	7.70	-2.8
Overnight	7.92	8.10	+2.3
2	1.98	1.90	-4.0
2	1.98	2.03	+2.5
Overnight	1.98	2.00	+1.0
Overnight	1.98	2.05	+3.5

APPLICATION TO BIOLOGICAL MATERIAL

Blood

Protein precipitation: to 2 ml. of distilled water in a centrifuge tube 1 ml. of blood is added and the solution well mixed; 3 ml. of 0.15N-Ba(OH)₂ are then added, followed by 3 ml. of 2.5% (w/v) ZnSO₄·7H₂O. The solution is well mixed, the tube stoppered and centrifuged. The clear supernatant liquid is used for estimating acetone, acetone plus acetoacetic acid and 'Total Acetone Bodies'.

Free acetone. A 3 ml. sample of the supernatant fluid is placed in the outer chamber of a greased Conway dish and the estimation carried out as above. Owing to dilution during precipitation and oxidation, it is necessary to multiply the results obtained from the standard graphs by the factor 9 to get the results in mg. acetone/100 ml. of original solution.

Free acetone can also be estimated without precipitation of protein if so desired. Recovery experiments which were carried out using the complete technique of precipitation etc., on standard solutions

in water and blood showed the same limits of accuracy as in direct estimation of standard solutions (Table 2).

Table 2. Recovery of added acetone

(The blank was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
Standard acetone solution		
7.92	7.80	-1.5
7.92	8.00	+1.0
7.92	8.10	+2.3
Standard solutions in blood		
7.92	8.01	+1.1
7.92	7.83	-1.1
7.92	8.10	+2.3
15.84	16.20	+2.3
Standard solutions in blood (direct method)		
7.92	8.00	+1.0
7.92	8.10	+2.3
7.92	8.20	+3.5
15.84	15.50	-2.1

Acetoacetic acid. Acetoacetic acid is determined by difference, the free acetone determined as above being subtracted from the acetone plus acetoacetic acid value, obtained as follows.

Acetone plus acetoacetic acid. A 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus and 1.1 ml. of 20N-H₂SO₄ and a glass bead are added. The apparatus is shut firmly after applying a very thin film of grease to the ground-glass joint, the flow of water is started and the contents of the reflux tube boiled for 5 min. The apparatus is then cooled rapidly under running water, tipped to mix well, and 3 ml. of the solution are transferred to the outer chamber of a Conway dish for the determination of the acetone present. The factor in this case is 12.3.

Recovery experiments were carried out as before, using commercial grade ethyl acetoacetate purified by distillation under reduced pressure. The results obtained (Table 3) came within $\pm 4\%$ of the theoretical amounts.

Table 3. Recovery of acetoacetic acid, expressed as acetone

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Standard solutions			
5.09	5.04	0	-0.9
5.09	5.04	0	-0.9
10.18	10.10	0	-0.8
10.18	9.84	0	-3.3
19.57	19.68	0	+0.6
19.57	19.68	0	+0.6
Standard solutions in blood			
5.09	5.16	0	+1.4
5.09	5.04	0	-0.9
5.76	18.45	12.92	-4.0
5.76	18.45	12.92	-4.0
11.52	24.60	12.92	+1.4
11.52	24.60	12.92	+1.4

Table 4. *Recovery of isopropanol, expressed as acetone*

Amount added (mg./100 ml.)	Microdistillation			Method of Friedmann (1938)		
	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Standard solution						
7.43	7.38	0	-0.6	7.25	0	-2.4
7.43	7.38	0	-0.6	7.50	0	+0.9
7.43	7.38	0	-0.6	7.25	0	-2.4
Standard solution in blood						
7.43	11.81	4.31	+0.9	7.5	0	+0.9
7.43	11.81	4.31	+0.9	7.25	0	-2.4
7.43	11.44	4.31	-4.0	7.25	0	-2.4

isoPropanol. *isoPropanol* is oxidized to acetone under the conditions normally employed for estimating 'Total Acetone Bodies' and so is included in this term. It can also be estimated separately using a modification of Friedmann's (1938) method in which the *isopropanol* alone is oxidized to acetone. A Markham steam distillation apparatus is used in this procedure, except in the case of blood, as it is more convenient for small quantities, as well as being quicker and easier to clean between samples.

A 50 ml. sample of blood is transferred to a large conical flask with 100 ml. of distilled water, 10 ml. of HgSO_4 solution (Friedmann, 1938) are added and the solution mixed, then 15 ml. of 10% (w/v) sodium tungstate and a little wax to prevent frothing. The solution is well shaken and steam-distilled, about 100–200 ml. of distillate being collected.

The distillate is washed into a round-bottomed flask, 5 ml. of HgSO_4 solution added and excess 10% (w/v) $\text{Ca}(\text{OH})_2$ suspension till the solution is alkaline. This solution is distilled directly into a conical flask, about 100 ml. of distillate being collected. 10 ml. of 20N- H_2SO_4 and excess $\text{K}_2\text{Cr}_2\text{O}_7$ are added to the distillate and the flask, loosely corked, is either placed on a drying oven (about 50°) for 2 hr., or left overnight at room temperature.

The solution is then washed into a round-bottomed flask and about 50 g. MgSO_4 added. The solution is distilled direct, a little less than 100 ml. distillate being collected; this is made up to 100 ml. with distilled water in a graduated flask and the acetone content determined by placing 3 ml. of the resulting solution in the outer chamber of a Conway dish and proceeding as above, using the factor 5 for the calculation.

Recovery experiments are shown in Table 4. The microrefluxing method used gave results similar to those obtained with Friedmann's method.

β -Hydroxybutyric acid. β -Hydroxybutyric acid is determined by difference, the acetone plus acetoacetic acid plus *isopropanol* values being subtracted from the value for 'Total Acetone Bodies', obtained as follows: a 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus with 0.7 ml. of 1.5% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ in 15.6N- H_2SO_4 , and a glass bead. The apparatus is closed firmly as before, the water flow started, and the contents boiled for 10 min. The solution is taken off the boil and 0.4 ml. of 10% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ run down the cold finger into the solution by means of a syringe. The apparatus is closed firmly again and the solution boiled for a further 10 min., cooled, tipped to mix the contents and 3 ml. transferred to

the outer chamber of a Conway dish and the acetone content determined. The factor here is 12.3.

The accuracy of the method for 'Total Acetone Bodies' when done on acetone and acetoacetic acid solutions was within $\pm 3\%$ (Table 5). Estimations

Table 5. *Recovery of 'Total Acetone Bodies'*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Acetone in standard solutions			
9.50	9.64	0	+1.5
9.50	9.32	0	-1.9
9.50	9.32	0	-1.9
23.76	23.37	0	-1.6
23.76	23.37	0	-1.6
23.76	23.99	0	+0.9
Acetone in blood			
9.50	12.97	3.20	+2.8
9.50	12.42	3.20	-2.9
9.50	12.67	2.95	+2.3
23.76	27.06	3.20	+0.4
23.76	27.68	3.20	+3.0
23.76	26.45	2.58	+0.4
Acetoacetic acid in standard solutions			
5.76	5.78	0	+0.3
11.52	11.56	0	+0.3
Acetoacetic acid in blood			
5.76	27.48	21.89	-3.0
11.52	33.33	21.89	-0.7

of β -hydroxybutyric acid in the quality obtainable, namely, British Drug Houses Laboratory Reagent, gave a practically consistent error of approx. 40% in both standard solutions and in blood (Table 6). Various modifications of this method which were tried, such as altering the concentration of chromic acid, varying the time of oxidation and varying the concentration of β -hydroxybutyric acid, all failed to give any increase in the percentage recovery. It was thought that part of this discrepancy might be due to impurity, but at our request Messrs British Drug Houses Ltd. kindly examined their product which we had used, the sodium salt of β -hydroxybutyric

acid, and reported that it gave the expected 78 % yield of acetone when examined by the method of Greenberg & Lester (1944) and that an approximate determination of purity by precipitation of the sodium with hydrochloric acid in ethanol, followed by the removal of the acid into ether, gave a result of about 98 %. A determination of the sodium content from sulphated ash also gave results equivalent to 98 % purity.

Table 6. *Recovery of β -hydroxybutyric acid, expressed as acetone*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Standard solutions			
5.08	3.07	0	39.6
5.08	3.07	0	39.6
5.08	3.08	0	39.4
10.15	6.13	0	39.6
10.15	6.13	0	39.6
10.15	6.26	0	38.3
15.24	9.10	0	40.3
15.24	9.10	0	40.3
15.24	9.35	0	38.6
Standard solutions in blood			
5.08	7.38	4.31	39.6
5.08	5.41	2.34	39.6
5.08	5.29	2.34	41.9
10.15	9.84	3.73	39.8
10.15	9.84	3.73	39.8
10.15	10.08	3.73	38.3
15.24	13.16	3.73	38.1
15.24	12.79	3.73	40.5
15.24	13.16	3.73	38.1

From our results it would appear, therefore, that chromic acid only oxidizes 60 % of the β -hydroxybutyric acid present to acetone under the above experimental conditions, as contrasted with the 78 % yield of acetone obtained by Greenberg & Lester (1944) using rather different conditions. As our method of determination yields results consistently 40 % low, then to determine the true amount of β -hydroxybutyric acid in a sample the value obtained by subtraction must be multiplied by 5/3. This corrected value added to the previously determined acetone plus acetoacetic acid plus isopropanol value will then give the true amount of 'Total Acetone Bodies' present.

Rumen contents

The rumen contents are strained through surgical gauze to remove large pieces of food, and the moderately clear liquid is used for the estimations. The protein is precipitated as for blood and the procedure and factors are the same except for isopropanol, where the factor is 5 and the procedure as follows: a 10 ml. sample of the filtered rumen liquor is placed in a Markham steam-distillation apparatus with 2 ml. of HgSO_4 solution (Friedmann, 1938) and 2 ml. 10% (w/v) sodium tungstate solution, and steam distilled,

about 100 ml. of distillate being collected. This is repeated with a further 10 ml. of liquid and the distillates added.

The combined distillates are washed into a round-bottomed flask and 5 ml. of HgSO_4 solution and excess 10% (w/v) $\text{Ca}(\text{OH})_2$ suspension added. This solution is distilled direct and the procedure then follows that given for blood.

Milk

As milk contains many reducing substances it is necessary to dilute the solution still further in order to get full oxidation of β -hydroxybutyric acid and isopropanol to acetone. This is achieved by precipitating the protein as follows: a 0.4 ml. sample of milk is added to 4.6 ml. of distilled water, 2 ml. of 0.15N- $\text{Ba}(\text{OH})_2$ solution and 2 ml. of 2.5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ are added. The tube is stoppered after mixing the contents and centrifuged as before.

The technique employed is the same as for blood except in the case of isopropanol where the method is similar to that used for rumen contents.

The factors are, for free acetone 22.5, for acetone plus acetoacetic acid and 'Total Acetone Bodies' 30.75 and for isopropanol 5.

Urine

The procedure and factors are the same as for blood except in the case of isopropanol, where the rumen technique is used, and the factor is 5.

In certain acute cases of acetonæmia, the content of 'Total Acetone Bodies' in urine rises to several hundred mg./100 ml. In these cases it is advisable to dilute the protein-free liquid still further before oxidation.

Recovery experiments carried out on milk and urine using added acetone gave the results shown in Table 7.

Table 7. *Recovery of added acetone from milk and urine*

(The blank value was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
Milk		
7.92	7.88	-0.5
7.92	7.88	-0.5
7.92	7.88	-0.5
15.84	16.20	+2.3
15.84	16.20	+2.3
Urine		
7.92	7.83	-1.1
7.92	7.83	-1.1
7.92	8.10	+2.3
15.84	15.93	+0.5
15.84	15.75	-0.5

Interference

The following substances were tested for interference in the estimation of acetone bodies: acetic acid, lactic acid, sodium chloride, cholesterol, urea, formaldehyde and acetaldehyde. With the exception of acetaldehyde, the results obtained showed there was no interference. This compound in concentrations as low as 3 mg./100 ml. reacts with the colour

reagent to give a slightly opaque orange solution. The interference is lessened a little after boiling with chromic acid, but not sufficiently to allow of an accurate determination even of total ketones in the presence of such amounts of this material.

Recovery from mixed solutions

From the preceding tables it can be seen that using standard solutions of one of the 'Acetone Bodies', the errors lay within the range $\pm 5\%$ except in the case of β -hydroxybutyric acid where the percentage yield of acetone was only 60, a discrepancy which is overcome by the use of the appropriate factor.

Experiments were carried out with similar small errors on standard mixtures of 'Acetone Bodies' after complete precipitation and oxidation (Table 8).

Estimations were also carried out on standard mixtures in blood and in rumen contents (Table 9), and the experimental errors were again found to lie within the same range. However, the concurrent experiments carried out using Denigès' method gave percentage errors with a much wider range.

DISCUSSION

From the results it can be seen that the method is readily applicable to two significant biological materials, namely, blood and rumen contents, in acetonaemia and pregnancy toxæmia. It can equally well be used for milk and urine with no appreciable change in precipitation and oxidation technique.

Although by no means perfect it is the first relatively simple method of obtaining a fairly adequate and reasonably accurate differentiation of the various ketone bodies involved in ruminant

Table 8. *Recovery of mixtures of ketone bodies using standard solutions*

Exp. no.	Mixture	Theoretical result (mg./100 ml.)	Amount found (mg./100 ml.)	Error (%)
1	Acetone	7.92	7.75	-2.1
	Acetoacetic acid	3.81	3.68	-3.4
	isoPropanol	7.43	7.50	+0.9
	β -Hydroxybutyric acid	5.08	5.30	+4.3
2	Acetone	7.92	7.75	-2.1
	Acetoacetic acid	3.81	3.81	0
	isoPropanol	7.43	7.15	-3.7
	β -Hydroxybutyric acid	10.15	9.99	-1.6
3	Acetone	15.84	15.30	-3.4
	Acetoacetic acid	7.62	7.45	-2.2
	isoPropanol	14.86	14.00	-5.7
	β -Hydroxybutyric acid	15.45	14.60	-5.5

Table 9. *Recovery of mixtures*

Exp. no.	Mixture	Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Amount recovered (mg./100 ml.)	Error (%)
In blood						
1	Acetone	7.92	7.87	0	7.87	-0.6
	Acetoacetic acid	3.81	3.63	0	3.63	-5.8
	isoPropanol	7.43	7.25	0	7.25	-2.4
	β -Hydroxybutyric acid	5.08	9.60	4.30	5.30	+4.3
2	Acetone	7.92	8.03	0	8.03	+1.0
	Acetoacetic acid	3.81	3.53	0	3.53	-7.0
	isoPropanol	7.43	7.25	0	7.25	-2.4
	β -Hydroxybutyric acid	10.15	18.90	8.20	10.70	+5.4
3	Acetone	15.84	15.30	0	15.30	-3.4
	Acetoacetic acid	7.62	7.46	0	7.46	-0.2
	isoPropanol	14.86	15.00	0	15.00	+0.9
	β -Hydroxybutyric acid	15.45	23.17	7.18	15.99	+3.5
In rumen contents						
4	Acetone	7.92	7.87	0	7.87	-0.6
	Acetoacetic acid	3.81	3.93	0	3.93	+3.2
	isoPropanol	7.43	7.25	0	7.25	-2.4
	β -Hydroxybutyric acid	5.08	8.08	3.17	4.91	-3.3
5	Acetone	15.84	15.50	0	15.50	-2.1
	Acetoacetic acid	7.62	7.10	0	7.10	-6.8
	isoPropanol	14.86	14.40	0	14.40	-3.1
	β -Hydroxybutyric acid	15.45	18.50	3.03	15.47	+0.1

pathology and should prove of value in throwing further light on the pathogenesis of various conditions in which acetonæmia is a prominent feature.

SUMMARY

1. A method has been devised for the estimation as acetone of the individual ketone bodies—acetone, acetoacetic acid, β -hydroxybutyric acid and isopropanol—within the range 0–120 mg. acetone/100 ml.

2. The basis of the method is the diffusion of acetone into an alkaline solution of salicylic aldehyde with the production of an orange-red colour, the intensity of which is measured in a photo-electric colorimeter.

3. The application of the method to biological materials such as blood, milk, urine and rumen liquor is described.

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The Preparation and Properties of β -Glucuronidase

3. FRACTIONATION AND ACTIVITY OF HOMOGENATES IN ISOTONIC MEDIA

By P. G. WALKER

Rowett Research Institute, Bucksburn, Aberdeenshire

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The first paper in this series (Kerr & Levvy, 1951) dealt with the fractionation of glucuronidase activity in water homogenates of mouse liver and other organs which is brought about by buffering to slightly acid pH. Changes in the glucuronidase activity of a tissue with the state of proliferation (Levy, Kerr & Campbell, 1948; Kerr, Campbell & Levy, 1949, 1950) were seen to be confined to the non-precipitable fraction. In the second paper (Walker & Levvy, 1951), it was shown that an identical fractionation of mouse-liver glucuronidase in untreated water homogenates could be achieved on the high-speed centrifuge, and that the sedimentable fraction was associated with cytoplasmic granules of all sizes. The use of water in preparing tissue homogenates caused pronounced osmotic swelling of the nuclei and other subcellular particles, leading sometimes to disruption. Homogenizing in isotonic media tends to preserve these bodies, and the present paper deals with the partition of glucuronidase activity between sedimentable material and the suspension fluid after homogenizing mouse liver in isotonic sodium chloride or sucrose solution. It was found that liver preparations did not display their full glucuronidase activity until a considerable proportion of the enzyme had been brought into solution.

METHODS

Preparation and fractionation of the enzyme. Unless stated to the contrary, the mouse liver was ground for 1 min. in the glass homogenizer (average speed 5000 rev./min.) with sufficient water, 0.17 M-NaCl or 0.25 M-sucrose solution to give a final tissue concentration of 10%, and samples were taken for assay. Following the procedure already described (Walker & Levvy, 1951) fractionation was then carried out either by centrifuging at a high speed (25000 g for 15 min.), or by adjusting the pH to 5.2 with acetate or citrate buffer (final concentration 0.1 N) and centrifuging at a low speed (1500 g for 15 min.). In the majority of experiments, the figure for sedimented enzyme activity was found by difference from the figures for total and unsedimented enzyme, the assumption being made that the latter was uniformly distributed between supernatant and precipitate. Control experiments to test this assumption, similar to those carried out with water homogenates (Walker & Levvy, 1951), showed that it also held for the homogenates in isotonic media.

Enzyme assay. After homogenizing liver in isotonic media, the enzyme associated with the subcellular particles did not display its full potential activity (see below). Unless otherwise stated, therefore, such homogenates were treated prior to assay by shaking with Ballotini, grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10 min. When the sedimented material from these homogenates was subjected to direct assay, it was found sufficient to rehomogenize in water.

The enzyme preparations, fractionated or unfractionated, were usually diluted to a final volume of 40 ml./g. moist tissue, and 0.5 ml. was transferred to a tube containing 0.5 ml. 0.01 M-phenolphthalein glucuronide solution (Talalay *et al.* 1946) and 3 ml. 0.1 N-acetate or citrate buffer, pH 5.2. The same buffer was used in the assay as had been used in fractionation. The remainder of the procedure followed that described by Kerr & Levvy (1951). Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μ g. phenolphthalein in 1 hr. at 37°.

Microscopy. As before (Walker & Levvy, 1951), preparations were examined under phase contrast.

RESULTS

Fractionation of homogenates in isotonic media

Table 1 shows typical results obtained for the fractionation of glucuronidase activity in adult and infant mouse liver homogenized in water, 0.17M-sodium chloride and 0.25M-sucrose solution. The

Table 1. *Fractionation of mouse-liver glucuronidase activity with acetate buffer after homogenizing in water or in isotonic solution*

(Insoluble enzyme activity found by direct measurement.)

Homogenizing fluid	G.U.* / g. liver		
	Total	Soluble	Insoluble†
Normal adult:			
Water	2900	1740	1140
0.17M-NaCl	2920	280	2600
0.25M-Sucrose	2850	220	2530
Infant (4 days old):			
Water	4810	3600	1240
0.17M-NaCl	4800	780	4020
0.25M-Sucrose	4720	720	4080

* For definition of G.U. see 'Enzyme assay'.

† Washed once on centrifuge with one-tenth volume of 0.1M-acetate buffer; wash fluid added to soluble fraction.

figures for normal adult were all obtained with samples of the same liver, whilst the infant figures refer to litter mates. Fractionation was carried out with acetate buffer, and in these particular experiments the figures for total, soluble and insoluble enzyme activity were all obtained by direct measurement. The results illustrate the following points. With either adult or infant liver, the total activities for all three types of preparation were in agreement. In each individual case, the figure for the total agreed within experimental error with the sum of the figures for the two fractions. There was, however, a striking difference between water homogenates on the one hand and homogenates in sodium chloride or sucrose solution on the other in the partition of enzyme activity between the two fractions.

In water the greater part of the activity was in a soluble form, but in the two isotonic media the soluble enzyme only accounted for 10–15% of the total activity. As already noted (Kerr & Levvy, 1951), the activity of the soluble fraction in a water homogenate of infant liver was higher than that in adult by an amount corresponding to the overall increase in activity. This was certainly not the case for homogenates in isotonic media, although the activity of the soluble fraction showed some increase in infants as compared with adults. Sodium chloride and sucrose did not act in these experiments as precipitating agents for enzyme soluble in water alone, but produced their effect by preserving some form of union between the enzyme and insoluble cell debris during homogenization. This was shown by experiments in which homogenates in water were subsequently made 0.17M with sodium chloride or 0.25M with sucrose. The partition of the enzyme remained characteristic of a water homogenate.

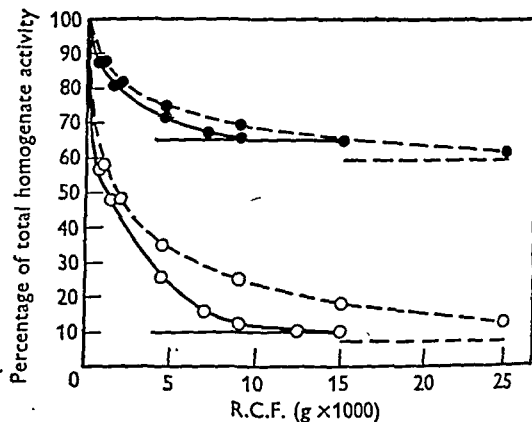


Fig. 1. Supernatant glucuronidase activity after centrifuging unbuffered homogenates of normal adult mouse liver at varying speeds for 15 min. Results expressed as percentages of the initial homogenate activity. O—O, liver homogenized in 0.17M-NaCl; ●—●, same liver homogenized in water and then made 0.17M with NaCl; O---O, liver homogenized in 0.25M-sucrose; ●---●, same liver homogenized in water and then made 0.25M with sucrose. The horizontal lines indicate the values for acetate soluble enzyme in the various preparations.

As with water homogenates (Walker & Levvy, 1951), the fractionation of glucuronidase activity produced by centrifuging at a high-speed unbuffered homogenates in isotonic media was identical to that produced by acetate buffer. This is shown in Fig. 1 for adult mouse liver. As a control for the effect of the homogenizing medium on the rate of sedimentation of subcellular particles, the tissue was also homogenized in water alone, and sodium chloride or sucrose was added before centrifuging. In each preparation, sedimentation on the high-speed centrifuge ceased when the figure for acetate

soluble enzyme was reached. At this stage, subsequent addition of acetate buffer to the supernatant caused no further separation of enzyme. As already noted (Walker & Levvy, 1951), sodium chloride caused agglutination and more rapid sedimentation of subcellular particles. Sucrose did not produce this effect. Considering either isotonic medium separately, it can be seen that the completeness of sedimentation of the insoluble enzyme at any given relative centrifugal force (R.C.F.) value was the same as in the appropriate control.

In spite of the higher overall glucuronidase activities of infant liver and liver regenerating after partial hepatectomy (Levy *et al.* 1948), the sedimentation of the insoluble enzyme in homogenates in either isotonic medium followed the same course as that shown in Fig. 1 for the normal adult tissue.

Microscopic observations

Sucrose homogenates. The components of a homogenate of mouse liver in 0.25M-sucrose resembled closely in appearance those described in similar preparations of rat liver (Hogeboom, Schneider & Pallade, 1948). The nuclei were smooth in outline and structureless, and granules of all sizes were discrete, uniformly dense and spherical. Complete sedimentation of insoluble enzyme was associated with the removal of all but a very few of the smallest visible particles from the suspension. Buffering the homogenate to pH 5.2 with acetate led to agglutination of all the visible components, and consequently much greater ease of sedimentation (*cf.* Walker & Levvy, 1951).

Saline homogenates. Compared with their appearance in sucrose homogenates, the nuclei in homogenates in 0.17M-sodium chloride were small, dark and granular, with well marked nucleoli. The granules were similar in appearance to those in sucrose homogenates, but were loosely agglutinated.

Water homogenates. As already noted (Walker & Levvy, 1951), the nuclei and granules showed pronounced osmotic swelling. The former were pale with faint nucleoli and were sometimes vacuolated. The latter were discrete, spherical and vesicular, with much less contrast than the granules in sucrose or saline homogenates.

On progressive dilution with water, the nuclei and granules in a saline or sucrose homogenate took on the appearance of those seen in water homogenates, without perceptible fragmentation of the larger granules. On making a water homogenate isotonic with sodium chloride or sucrose, however, the particles did not revert completely to the appearance typical of a homogenate prepared in the isotonic medium. Most of the granules became smaller and darker, but irregular in outline, while the nuclei became slightly crenated.

Factors influencing the partition of enzyme activity in the homogenate

It would appear from the experiments described above that the greater part at least of the soluble glucuronidase activity in a water homogenate of mouse liver is not originally free in the cytoplasm of the intact cell, but is liberated from the insoluble particles during the preparation of the homogenate. The effects were studied of three variables which might conceivably influence the final partition of enzyme activity after grinding the tissue in the glass homogenizer, namely, the temperature of the homogenizer, the period of homogenizing and the tonicity of the medium.

Temperature. The freshly dissected, chilled tissue was homogenized with cold 0.25M-sucrose solution for 1 min. in a glass homogenizer surrounded with crushed ice. The centrifuge tubes were also surrounded with ice during the subsequent fractionation using the acetate buffer method. Results obtained in this way were identical with those obtained for another sample of the same liver put through the same treatment without temperature control. This was also true for a third sample of liver homogenized at 37° and then fractionated in the usual way.

Period of homogenizing. Portions of adult liver were homogenized for varying periods, without temperature control, and fractionated by buffering with acetate. The results reveal a progressive release of enzyme from the particles to the solution as homogenizing was prolonged (Table 2). The time factor was least marked in water. There was no inactivation of the enzyme in these experiments.

Table 2. *Fractionation of adult mouse-liver glucuronidase activity after grinding in the glass homogenizer for varying periods with water, 0.17M-NaCl or 0.25M-sucrose solution*

Period of homogenizing (min.)	Acetate-soluble enzyme (% total activity)		
	Water	0.17M-NaCl	0.25M-Sucrose
0.5	64	9	7
1	66	11	8
2	70	15	15
3	73	19	20
5	75	26	25

An attempt was made to reduce the figure for soluble enzyme activity in an isotonic preparation below the lowest given in Table 2. Liver was ground in a chilled mortar with 0.25M-sucrose solution and strained through bolting silk. The preparation thus obtained was very similar in its microscopic appearance to the usual sucrose homogenate, and had the same percentage soluble enzyme activity as the 0.5 min. homogenate (Table 2).

Tonicity. Fig. 2 shows the partition of enzyme activity after homogenizing adult liver for 1 min. in varying concentrations of sucrose. No change in the value for acetate-soluble enzyme was observed in passing from the isotonic to hypertonic medium, but as the medium was made increasingly hypotonic there was a progressive rise in the activity of this fraction until the figure for water was reached. The overall glucuronidase activity of the preparation did not vary with the concentration of sucrose.

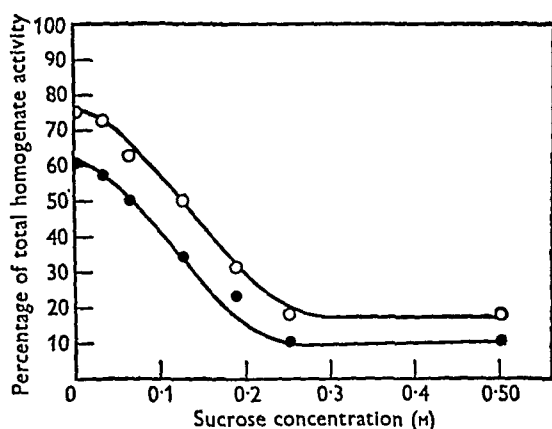


Fig. 2. Acetate-soluble glucuronidase activity in mouse-liver preparations after homogenizing in varying concentrations of sucrose. Results expressed as percentages of the total homogenate activity. ●—●, adult; ○—○, 4-day-old.

Fig. 2 also shows that at all concentrations of sucrose, infant liver had a greater proportion of the enzyme in a soluble form than the adult tissue. The difference became more marked with decreasing concentration of sucrose till in water alone, as already noted, it accounted entirely for the higher overall activity of the infant tissue. Similar curves were obtained for liver homogenized in sodium chloride solutions covering the same range of tonicity.

Measures causing the release of enzyme from insoluble material

Dilution with water. On dilution with water, enzyme was released from the particles in homogenates prepared in isotonic sucrose or sodium chloride solution, to give a distribution identical to that obtained by homogenizing directly in media of the same final tonicity. The change in the distribution of the enzyme appeared to be instantaneous.

Incubation at 37°. It was previously found for water homogenates (Kerr & Levvy, 1951; Walker & Levvy, 1951) that incubation in acetate buffer, pH 5.2, at 37° for 4 hr., converted all the enzyme present into a soluble form, with little change in the microscopic appearance of the insoluble particles. This property of acetate was not shared by citrate,

nor did release of enzyme from the particles occur on incubating unbuffered preparations.

Homogenates in 0.17 M-sodium chloride or 0.25 M-sucrose solution behaved like water homogenates in that incubation for 4 hr. after buffering to pH 5.2 with acetate (final concentration 0.1 N) without change in tonicity resulted in the conversion of enzyme into a soluble form, although conversion was not so complete as in water. When acetate was replaced by citrate buffer (final concentration 0.1 N)

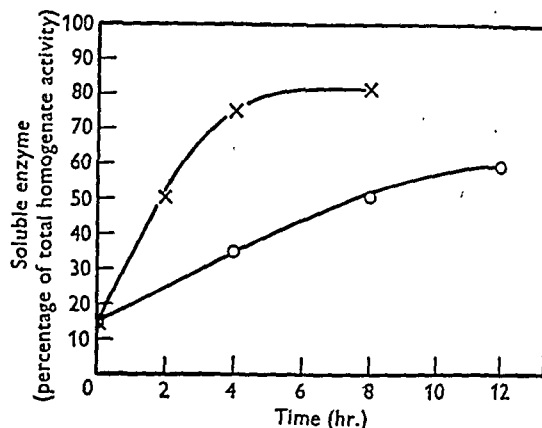


Fig. 3. Changes in the partition of the glucuronidase activity in a homogenate of adult mouse liver in 0.17 M-NaCl solution after incubating at 37° for varying periods. Acetate-soluble enzyme expressed as a percentage of the total homogenate activity. ○—○, unbuffered homogenate; ×—×, homogenate made 0.1 N with respect to acetate buffer, pH 5.2.

at pH 5.2, or when the unbuffered preparation was incubated at its natural pH (6.5–6.8), the release of enzyme from the particles was much slower and tended to a maximum at the figure for soluble enzyme in a water homogenate. This is illustrated for a saline homogenate in Fig. 3. In the unbuffered preparations incubation resulted in a change in the microscopic appearance of the particles, resembling that observed when homogenates in isotonic media were diluted with water (see 'Microscopic observations'). No enzyme inactivation was observed in these experiments.

Mechanical disintegration. As previously noted for water homogenates (Walker & Levvy, 1951), shaking with Ballotini in the Mickle tissue disintegrator caused almost complete release of the enzyme from the particles in a homogenate in isotonic sodium chloride or sucrose solution, without appreciable inactivation. The Waring Blender has been found to act similarly in this respect (Table 3), practically all the enzyme in liver or a liver homogenate being converted to a soluble form in 3 min., without any loss in overall activity. At this stage, all the components of the preparation were seen under the microscope to have undergone fragmentation, with complete loss of structure.

It has been customary to use the supernatants from water homogenates for the assay of tissue glucuronidase activity (see Kerr & Levvy, 1951). It can be seen from Table 3 that the use of the Waring Blender instead of the glass homogenizer can lead to a very different value for the glucuronidase activity of a tissue in such methods of assay (cf. Fishman & Talalay, 1947).

Table 3. *Fractionation of adult mouse-liver glucuronidase activity after treatment for varying periods in the Waring Blender*

(The starting material consisted of liver which had previously been ground in water, 0.17M-NaCl or 0.25M-sucrose solution in the glass homogenizer for 1 min., or the whole organ to which water was added in the blender.)

Period of treatment (min.)	Acetate-soluble enzyme (% total activity)			
	Water homogenate	Saline homogenate	Sucrose homogenate	Liver and water
0	57*	14	14	—
0.5	71	77	72	—
1	88	89	86	79*
2	95	96	93	90
3	100	100	98	98
5	100	100	100	100

* These figures give a direct comparison of liver treated with water for 1 min. in the glass homogenizer and the Waring Blender respectively.

Freezing. As in water homogenates (Walker & Levvy, 1951), the particles in sucrose or saline homogenates of liver gave up their glucuronidase activity to the solution on repeated freezing with a mixture of solid carbon dioxide and acetone, and thawing at 37°. The rates of transfer were, however, very different in the two types of isotonic homogenate. In sodium chloride solution, 60% of the enzyme activity became soluble after a single freezing, whereas four freezings were necessary before this stage was reached in sucrose solution.

Treatment with acetone. After homogenizing mouse liver in acetone (Walker & Levvy, 1951) followed by resuspension of the dry powder in water, 0.17M-sodium chloride or 0.25M-sucrose solution, almost all the enzyme activity was soluble.

Treatment with surface-active agents. The actions of Teepol XL (British Drug Houses Ltd), Triton X-100 and Triton A-20 (Rohm and Haas Co.) on homogenates in isotonic media were identical in all respects with their actions on water homogenates (Walker & Levvy, 1951). The first two agents in suitable concentrations caused complete conversion of the enzyme into a soluble form, but Teepol XL differed from Triton X-100 in that high concentrations were inhibitory. Triton A-20 had no effect of any kind.

Distribution of the enzyme according to particle size

Fractional sedimentation of mouse-liver homogenates in 0.25M-sucrose solution was carried out by a procedure which followed that of Schneider & Hogeboom (1950) as closely as our equipment permitted.

Fraction 1. A 10% homogenate (10 ml.) was centrifuged for 10 min. at 700 g and the supernatant was decanted. The residue was resuspended in 8 ml. isotonic sucrose solution and sedimented as before. The debris, consisting of nuclei contaminated with erythrocytes, unbroken liver cells and cytoplasmic granules, was resuspended for assay.

Fraction 2. The combined supernatants from fraction 1 were centrifuged at 5000 g for 10 min., and the supernatant was decanted and again centrifuged at 5000 g for 10 min. Two washings of the combined sediments were carried out by suspending the solid in 5 ml. sucrose solution and centrifuging at 20 000 g for 10 min. The debris, a fairly homogeneous preparation of the larger cytoplasmic granules, was finally resuspended for assay.

Fraction 3. Values for this fraction, consisting of all remaining insoluble material in the preparation, were found by difference, since centrifuging at 25 000 g (the maximum r.c.f. value at our disposal) did not give a sufficiently well packed sediment for clean separation. The supernatant and washings from fraction 2 were combined and aliquots were taken for assay. The figures thus obtained and those for fraction 4 gave by difference the figures for fraction 3.

Fraction 4. The remainder of the preparation was centrifuged at 25 000 g for 20 min. and the supernatant was decanted. After recentrifuging at the same speed, samples of the now optically clear supernatant were withdrawn directly from the centrifuge tube for assay of the 'soluble enzyme' in the homogenate.

Results for representative experiments are shown in Table 4. The main points emerging from this table are as follows. The total enzyme activity of the homogenates was fully accounted for by the activities of the separated fractions, and 80–90% was sedimentable. Of this, four-fifths was in the pure granule fractions, which also showed the highest specific activities. The relatively low activity of the impure nuclear fraction may well have been due to the contaminating granules.

Infant liver and liver regenerating after partial hepatectomy showed a similar distribution of insoluble enzyme activity to that seen in the normal adult tissue. The higher total and specific activities of growing and regenerating liver (cf. Levvy *et al.* 1948) were reflected in every centrifuge fraction.

All fractions of the sedimentable material showed approximately equal (60–70%) release of enzyme

activity to the solution on dilution with water. Under the microscope, there was no perceptible fragmentation of the particles in fraction 2. In infant as compared with adult liver a greater percentage of the enzyme in every fraction (70–80%) was converted to the soluble form.

in a given preparation no matter which method of treatment was adopted, but the results make it clear that it was unnecessary to take more than part of the enzyme into solution before the preparation displayed its full potential activity. The release of enzyme activity from insoluble material was readily

Table 4. *Fractional sedimentation of unbuffered mouse-liver homogenates in 0.25M-sucrose solution*

Fraction no. and description	N (total)		Enzyme activity		
	mg./g. liver	% total	G.U.* /g. liver	% total	G.U.* /mg. N
Normal adult liver					
Total homogenate	39.0	100	3580	100	92
1. Nuclei, etc.	7.5	19	510	14	68
2. Large granules	6.5	17	1100	31	170
3. Small granules	6.8	17	1430	40	210
4. Soluble enzyme	16.5	42	470	13	29
Sum of fractions	37.3	95	3510	98	—
Infant liver (4-day-old)					
Total homogenate	31.0	100	5100	100	164
1. Nuclei, etc.	6.5	21	750	15	117
2. Large granules	5.1	16	1310	26	256
3. Small granules	5.3	17	2000	39	377
4. Soluble enzyme	14.2	46	950	19	67
Sum of fractions	31.1	100	5010	99	—
Regenerating liver (4 days after partial hepatectomy)					
Total homogenate	36.6	100	4480	100	122
1. Nuclei, etc.	7.2	20	550	12	76
2. Large granules	7.0	19	1360	30	194
3. Small granules	5.9	16	1820	41	309
4. Soluble enzyme	17.0	46	820	18	48
Sum of fractions	37.1	101	4550	101	—

* For definition of G.U. see text.

The activity of the enzyme in homogenates in isotonic media

Kerr & Levvy (1951) found that the use of too high a tissue concentration in the assay of water homogenates of mouse liver led to false low results for the total glucuronidase activity. Full activity was shown at all concentrations once the enzyme had been completely converted into a soluble form by incubation in acetate buffer at 37° for 4 hr.

Table 5 shows that tissue concentration had a similar effect in the assay of homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The activity no longer varied with tissue concentration once the enzyme had been completely converted into a soluble form. Even at low concentrations, however, homogenates in isotonic media, unlike those in water, increased considerably in activity when the enzyme was made soluble.

Table 6 shows parallel figures for the total glucuronidase activity and the percentage soluble enzyme activity in homogenates of adult mouse liver after treatment by various methods which cause the transfer of enzyme activity from insoluble material to the solution. The same final activity was reached

controlled when it was brought about by dilution with water or incubation in acetate buffer, and with these measures full activity was reached when only about half the enzyme had gone into solution, i.e. when the distribution of enzyme activity had become similar to that seen in water homogenates. Whereas these measures released the enzyme by a gradual process affecting all insoluble particles equally, mechanical disintegration in the tissue disintegrator or the Waring Blendor resulted in complete destruction of individual particles and thus did not produce complete activation in the present experiments until nearly all the enzyme had been brought into solution. The release of enzyme activity from insoluble material by the surface-active agents Triton X-100 and Teepol XL was difficult to control in degree, but the figures given in Table 6 show that when this process was complete the enzyme was fully active.

Since in the assay the incubation with substrate was carried out in acetate buffer, in which activation of the enzyme must have occurred, the figures given in Table 6 for the total glucuronidase activity of homogenates are subject to error. This consideration does not apply to the figures for soluble enzyme

activity, this fraction of the enzyme being already fully active.

The error arising from the activation of the enzyme during the incubation with substrate is illustrated in Fig. 4 by velocity curves for the hydrolysis of phenolphthalein glucuronide in acetate buffer by mouse liver homogenized in 0.17M-sodium chloride. When the homogenate (2.5%,

activity was slower, and full activity was only reached after 90 min. incubation. This concentration of sodium chloride did not inhibit fully activated preparations of the enzyme.

Similar results were obtained in experiments with homogenates in isotonic sucrose solution, except in that activation proceeded more slowly than in sodium chloride solution. Sucrose in a final

Table 5. *Variations in the glucuronidase activity of adult mouse liver with the homogenate concentration taken for assay*

(All results refer to a single sample of tissue; activities were measured in acetate buffer before and after conversion of all the enzyme into a soluble form by treatment for 20 min. in the tissue disintegrator.)

Concentration of homogenate* (g. tissue/100 ml. homogenizing fluid)	Glucuronidase units/g. liver			
	Homogenate in 0.17M-NaCl		Homogenate in 0.25M-sucrose	
	Before treatment	After treatment	Before treatment	After treatment
40	1320	3200	1200	3240
20	1680	3200	1560	3200
10	2200	3200	2120	3240
5.0	2400	3280	2240	3200
2.5	2380	3240	2200	3240
1.0	2300	3200	2100	3240

* 0.5 ml. of homogenate of the concentration shown was incubated with 0.5 ml. substrate solution and 3 ml. buffer in the usual way.

Table 6. *Effects of various measures on the total and soluble glucuronidase activity in homogenates (2.5%, w/v) of mouse liver in isotonic NaCl or sucrose solution*

(Activities measured in acetate buffer, and results expressed as percentages of the activity of the whole homogenate after 10 min. treatment in the tissue disintegrator.)

Treatment		Homogenate in 0.17M-NaCl		Homogenate in 0.25M-sucrose	
Agent	Extent	Total activity	Soluble activity	Total activity	Soluble activity
None	—	75	12	66	8
Tissue disintegrator	1 min.	82	50	79	55
	2 min.	91	72	88	78
	5 min.	100	98	100	97
	10 min.	100	100	100	100
Waring Blendor	0.5 min.	88	68	87	66
	1 min.	94	85	95	83
	3 min.	100	100	101	100
	5 min.	99	99	101	103
Triton X-100	0.1 %	101	101	102	102
Teepol XL	0.4 %	101	98	101	97
Water	$\frac{1}{3}$ vol.	82	14	75	17
	1 vol.	90	23	86	29
	3 vol.	100	47	97	51
Incubation in acetate buffer, pH 5.2	1 hr.	87	26	83	18
	2 hr.	92	41	91	30
	4 hr	100	80	100	69

w/v) was added to the usual buffer-substrate mixture, the rate of hydrolysis gradually increased until, after about 45 min., it reached the value obtained with a fully activated sample of the same homogenate. When the buffer-substrate mixture was made 0.17M with respect to sodium chloride, so as to prevent dilution of the homogenizing medium in the enzyme preparation, the change in enzyme

concentration of 0.25M was found to cause about 10 % inhibition of fully activated glucuronidase preparations.

Replacing acetate by citrate buffer during the incubation at pH 5.2 not only slowed down the transfer of enzyme activity from insoluble material to the solution (see above), but also decreased the rate of activation of homogenates in isotonic media.

The rate of activation in citrate buffer became even slower when 0.17M-sodium chloride was present, and slower still when this was replaced by 0.25M-sucrose. Velocity curves for the hydrolysis of phenolphthalein glucuronide in sucrose-citrate (pH 5.2) by homogenates of adult and infant mouse liver in 0.25M-sucrose solution are shown in Fig. 5.

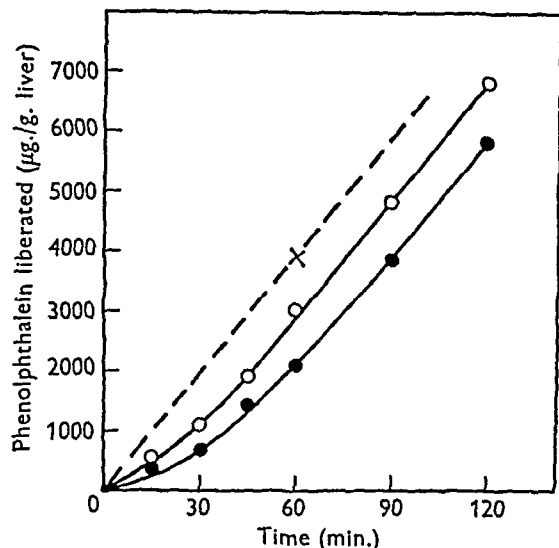


Fig. 4. Rate of hydrolysis of phenolphthalein glucuronide (0.00125M) in acetate buffer (final concentration 0.075N, pH 5.2) by a homogenate of adult mouse liver in 0.17M-NaCl solution. Final tissue concentration 0.3% (w/v). ●—●, buffer and substrate made 0.17M with respect to NaCl; ○—○, no NaCl added to buffer or substrate; —x—, assay of homogenate after treatment for 10 min. in the tissue disintegrator.

This graph illustrates the difficulties which would attend attempts to make comparative measurements of mouse-liver glucuronidase activity in homogenates in isotonic media without preliminary activation. It can be seen, however, that in untreated preparations the activity of the infant tissue was at all stages higher in terms of µg. phenolphthalein liberated/g. liver than that of the adult tissue (cf. Levvy *et al.* 1948).

chloride solution was incubated with increasing concentrations of phenolphthalein glucuronide in acetate buffer, inhibition of glucuronidase activity by excess substrate was seen, whether or not the enzyme preparation had been subjected to prior activation. Considerable activation of the untreated preparation must of course have occurred during the assay in acetate buffer. When a homo-

The nature of the inactive enzyme

Table 7 shows the effect of varying the substrate concentration on the activity, expressed as glucuronidase units, of different preparations of the same tissue added to incubation mixtures of varying composition. When a homogenate in 0.17M-sodium

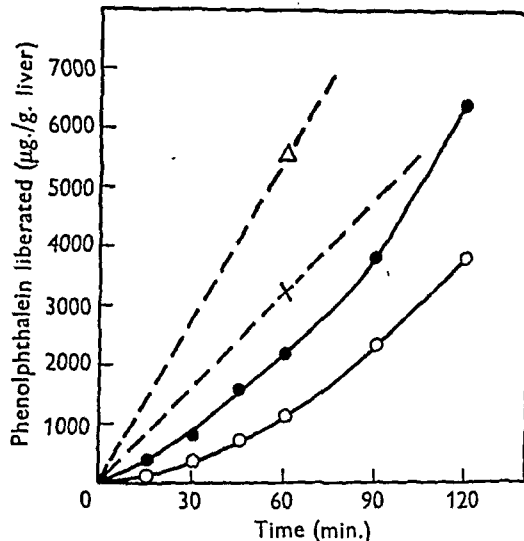


Fig. 5. Rate of hydrolysis of phenolphthalein glucuronide (0.00125M) in citrate buffer (final concentration 0.075N) by homogenates of adult and infant mouse liver in 0.25M-sucrose solution. Final tissue concentration 0.3% (w/v). Substrate and buffer made 0.25M with respect to sucrose. ○—○, adult; ●—●, infant (4-days-old); —x—, assay of adult liver after treatment of homogenate for 10 min. in the tissue disintegrator. —△—, assay of infant liver after treatment of homogenate for 10 min. in the tissue disintegrator.

Table 7. Effect of varying substrate concentration on the rate of hydrolysis of phenolphthalein glucuronide in 0.1N-acetate or citrate buffer by homogenates of adult mouse liver in 0.17M-NaCl or 0.25M-sucrose solution

(All results refer to a single sample of tissue; g.u./g. liver measured at pH 5.2 before and after treatment for 10 min. in the tissue disintegrator.)

Substrate concentration (M)	Saline homogenate in acetate buffer		Sucrose homogenate in citrate buffer		Sucrose homogenate in sucrose-citrate buffer	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
0.00125	2640	3600	2000	3600	1320	3200
0.00438	2320	3120	1960	3040	1440	2640
0.00875	2000	2480	2000	2400	2080	2240

genate in 0.25M-sucrose solution was added to citrate buffer containing the same concentration of sucrose and a varying concentration of substrate, increase in the latter caused a rise in the net activity of the untreated homogenate, but a fall in the activity of the activated preparation. Whatever the factor may be that is responsible for the low activity of glucuronidase in mouse liver homogenized in isotonic media, it can thus to some extent be overcome by the use of a substrate concentration in itself high enough to inhibit the enzyme. These two opposing effects of increasing the substrate concentration on the net activity of an untreated preparation were exactly balanced when a sucrose homogenate was incubated with a mixture of citrate buffer and phenolphthalein glucuronide made up in water alone.

The results shown in Table 7 are entirely explicable on the view that the enzyme in a homogenate of mouse liver in isotonic media, but not in water, is subject to the action of an endogenous competitive inhibitor. An alternative explanation must, however, be considered, namely that the enzyme associated with the insoluble particles is surrounded by material which the substrate only penetrates with difficulty.

DISCUSSION

Accepting that the localization of an enzyme in the intact cell can be inferred from its distribution amongst the components of an isotonic homogenate, it is concluded from the present study that all or almost all of the glucuronidase activity of mouse liver is associated *in vivo* with subcellular particles, especially the large and small cytoplasmic granules.

The enzyme can be irreversibly released from the particles by various treatments which produce either swelling or fragmentation of the particles. The small amount of the enzyme which is present in a soluble form in isotonic preparations may be derived from the particles when they are liberated into the medium or it may arise from damage to the particles in the homogenizer. The amount of this soluble fraction is, however, not reduced either by employing hypertonic media which preserve better the intracellular appearance of the particles (Hogeboom *et al.* 1948) or by a very gentle grinding of the tissue. As the medium is made increasingly hypotonic there is a transfer of enzyme activity from the particles to the solution till, at low sucrose or saline concentrations, the distribution characteristic of a water homogenate is approached. It may be significant that the release of enzyme activity which occurs on prolonged incubation of a homogenate in an isotonic medium also ceases when the soluble activity reaches the figure for a water homogenate.

The distribution of glucuronidase in infant and regenerating liver is similar to that in normal adult liver, and the higher enzyme activity of these tissues is spread over particles of all sizes. In actively proliferating as compared with resting tissue, a greater proportion of the enzyme in all fractions of the sedimentable material is released in water. This agrees with the observation of Kerr & Levvy (1951) who found that in water homogenates the increase in the soluble activity accounted entirely for the higher overall activity of proliferating tissues.

As enzyme is released from the particles in homogenates in isotonic media, there is an increase in the activity of the enzyme remaining bound to insoluble material. Two explanations have been suggested for the initially low glucuronidase activity of these homogenates, namely the existence of a competitive inhibitor in the homogenate, or of material which hinders access of the substrate to the enzyme in the insoluble particles. The latter explanation is considered to be the more satisfactory since it covers the behaviour of water homogenates. In hypotonic media, a change in the particle occurs, permitting the escape of some of the enzyme from the particles. This change presumably permits much easier access of the substrate to the enzyme remaining within the particles. The existence of a competitive inhibitor is hard to imagine since it would have to be very selective in its action, affecting the insoluble enzyme in homogenates in isotonic media, but neither the enzyme in completely soluble preparations, nor the insoluble enzyme fraction in water homogenates.

Various factors have been found to influence results obtained in the assay of glucuronidase associated with insoluble material. It is clear that reliable comparative measurements of tissue activity can only be made after complete solution or complete activation of the enzyme. The physiological significance of results thus obtained is, however, another matter. If one accepts the premise that the state of an enzyme in homogenates in isotonic media approximates to its state in the intact tissue (Schneider & Hogeboom, 1951), the possibility must be considered that factors such as those encountered in the present work have effects on the activity of the enzyme in living tissues, which may be as important as changes in the active protein content, detected by the use of soluble preparations.

SUMMARY

1. The partition of glucuronidase activity between the subcellular particles and the solution has been studied in homogenates of mouse liver in isotonic sodium chloride or sucrose solution. The particles were sedimented by centrifuging at a high speed, or by centrifuging at a low speed after agglutination with acetate buffer.

2. The two methods of sedimentation gave identical results and 80–95 % of the enzyme activity was found in the precipitate.

3. As the medium was made increasingly hypotonic, a greater proportion of the enzyme activity was released from the particles. Particles of different sizes, separated by fractional sedimentation, behaved comparably in this respect.

4. The distribution of enzyme activity over particles of different sizes was the same in infant liver and in liver regenerating after partial hepatectomy as in the normal adult tissue, but prepara-

tions of the growing or regenerating tissue showed greater release of enzyme activity to the solution on dilution with water.

5. The homogenates in isotonic media did not display their full glucuronidase activity on direct assay, but partial activation occurred under assay conditions. Full activity was displayed when about half the enzyme present was brought into solution.

6. The possible mechanism of activation and its physiological significance are discussed.

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The Chemical Composition and Structure of the Yeast Cell Wall

BY D. H. NORTHCOTE

Biochemical Laboratory, University of Cambridge

AND R. W. HORNE

Cavendish Laboratory, University of Cambridge

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The chemical composition of the yeast cell wall was first studied by Salkowski (1894), who investigated the polysaccharide material which remained after intact cells had been digested with dilute sodium hydroxide solution. Zechmeister & Tóth (1934) continued the study, but again disrupted the cells by fairly vigorous chemical action. They suggested, however, that an enzymic method might do less damage to the cell wall, and indeed later they isolated the glucan component of the cell wall by the action of pepsin and amylase on an autolysed yeast suspension (Zechmeister & Tóth, 1936). These chemical and enzymic investigations have indicated that several polysaccharides may be present in the cell wall, and it has been suggested that, besides the glucan, a mannan (Haworth, Hirst & Isherwood, 1937) and possibly a 'glycogen' (Ling, Nanji & Panton, 1925) may form part of the structure; no direct evidence for this has been given. The structure of these polysaccharides has been investigated by

various workers. The general method of isolation has been extraction of whole yeast with 3 % sodium hydroxide solution at 100°, whereby the mannan and some glycogen go into solution, whereas the glucan and most of the glycogen remain as the insoluble material, which is seen under the microscope to retain the shape of the cell, and is therefore assumed to be part of the cell wall. The glucan was shown to contain only glucose with β -1:3 linkages between the radicals, by Zechmeister & Tóth (1934) and Hassid, Joslyn & McCready (1941); more recently Bell & Northcote (1950) found that this polysaccharide was highly branched and determined its average chain length. The structure of the mannan has been studied by Haworth *et al.* (1937), Haworth, Heath & Peat (1941) and Lindstedt (1945), but the constitution of the glycogen, and especially its possible existence in two forms (Ling *et al.* 1925; Daoud & Ling, 1931) has received little attention.

In the present work the yeast cell wall has been isolated in two ways:

- A. By physical methods entailing mechanical breakage of the cell and isolation of the washed cell wall by differential centrifugation.
- B. By chemical methods, entailing breakage and isolation, similar to those used by the previous workers, i.e. digestion of the whole cell with 3% sodium hydroxide.

In this way a more complete and quantitative survey of the substances making up the structural elements of the outer cell wall has been obtained than was hitherto possible. Both a chemical and a microscopical examination of the preparation have been made and in this latter connexion the material has been found to be very suitable for investigation by the electron microscope.

EXPERIMENTAL

Material used and general analytical methods

The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers' Co. Ltd.). Its dry weight, determined under the same conditions as those used with the cell wall preparations, was 27.3% of the moist weight.

All the analyses were carried out on material dried at 0.01 mm. Hg over P_2O_5 at room temperature.

Total N (Kjeldahl) was determined using Nessler's reagent (Umbreit, Burris & Stauffer, 1949). Total P was determined according to Fiske & Subbarow (1925, cf. Umbreit *et al.* 1949).

Chromatography of sugars. Descending chromatograms were run on Whatman no. 1 papers with *n*-butanol/water at 37° for 60 hr. (Hough, Jones & Wadman, 1950). Glucose, galactose, glucosamine, mannose and arabinose were used as markers. The spots were coloured with aniline hydrogen phthalate (Partridge, 1949) and ammoniacal $AgNO_3$ on duplicate papers.

Chromatography of amino-acids. Descending chromatograms were run with phenol/0.3% (w/v) aqueous NH_3 at 20° in an atmosphere of coal gas (Consden, Gordon & Martin, 1944). The spots were coloured with 0.3% (w/v) triketohydrindene hydrate in *n*-butanol.

The solutions applied to the chromatograms were adjusted so that the approximate concentration of the sugars and the amino-acids was never less than 1%.

The work below is described in two parts, dealing with cell wall material obtained by mechanical breakage of the cell, preparation A, and that obtained by chemical cytolysis of the cell, preparation B.

PREPARATION A

Isolation of the cell wall material by mechanical breakage of the cell

Yeast (700 mg.) was suspended in 10 ml. of water with 4 g. of fine glass beads (Ballotini no. 12, Chance Bros Ltd., Birmingham) and the mixture placed in a vertical cup (internal measurements 5 × 2.2 cm.) of a Mickle cell disintegrator (Mickle, 1948). Vibration was continued for 30 min., after which the resultant suspension was decanted away

from the glass beads. The supernatant was centrifuged at 1500 g for 10 min. (height of tube 10 cm.), and the residue washed repeatedly with water until the final supernatant became clear; the residue comprised the required cell wall fraction. The initial supernatant and the washings were combined and centrifuged at 14000 g for 20 min. (height of tube 10 cm.), when a residue of fine particles was obtained. The final supernatant and the two residues were freeze-dried. The yields of material from three representative fractionations are shown in Table 1. The beads cannot have contributed much to these fractions since their ash content never exceeded 3%.

Table 1. *Yield of fractions obtained by differential centrifugation of mechanically disintegrated yeast cells*

Exp. no.	mg./100 mg. dry wt. taken			Recovery (%)
	Residue deposited at 1500 g (cell wall fraction)	Residue deposited at 14000 g (small particle fraction)	Supernatant ('soluble' material)	
1	15.3	30.1	44.4	89.8
2	14.1	28.4	41.8	84.3
3	14.5	30.3	42.0	86.8

Microscopical examination of cell wall

The cell walls isolated in the above manner are Gram-negative, whereas the whole yeast cells are Gram-positive. It is thus an easy matter to distinguish any unbroken cells in the preparations by a microscopical examination of stained films. Many fields of numerous preparations were examined and in no case was a whole cell detected. The cell walls were also examined by the phase-contrast microscope and here again no whole cells and very little debris could be detected. It is concluded that the material isolated does represent solely the outer membranes of the cell, and that chemical investigations carried out on it will in fact characterize the cell wall which is thought to be made up of these membranes.

Electron microscope. A comprehensive examination of the cell wall was made under the electron microscope using both Siemens and Radio Corporation of America (R.C.A.) instruments. The high tension voltages used ranged from 50 to 90 kV. The material was suspended in water and allowed to dry at room temperature in air on the filmed specimen grids. Films of two types were used as supporting membranes, these being mounted on standard Kodak copper grids. Early preparations were mounted on nitro-cellulose supporting films, but owing to the relatively large size of yeast cells these films frequently ruptured during investigation in the microscope. 'Formvar' (polyvinyl formal) films prepared from dioxan solution were found to be much stronger, particularly when large numbers of cells were present in the field under examination. Preparations were shadowed with an alloy of Au and Pd employing the usual shadow-casting techniques. The shadowing angle was 45°.

The most characteristic and obvious detail in the structure of the cell walls was the occurrence of scars on the surface; these appeared on the majority of the larger cells but not on the smaller cells. Some cells carried as many as sixteen such markings (Fig. 4a). By comparisons with those cells to which were attached parts of the cell wall of the bud, and

with that of an intact cell in the process of budding (Fig. 1), these markings were identified as budding scars and appeared as circular thickenings of the cell surface (Figs. 2 and 4a-d). The figures are metal-shadowed electron micrographs. The bud scars could also be seen on the cell walls under the phase-contrast microscope. It is of interest to note that Dorsten, Oosterkamp & Le Poole (1947) used whole yeast cells for testing the 400 kV. experimental electron microscope and the pictures taken by their instrument show indications of these budding scars in the whole cell. When the preparations were treated with 2N-HCl the scars appeared to be more resistant to chemical attack than the general cell surface. By observations of the edge of the cells and of the apparent peeling of the membranes which often occurred at the bud scar (Fig. 2) it could be seen that the cell envelope consisted of at least two membranes. When the preparations were extracted successively with methanol and ether (see below) to remove lipids, and re-examined, the division into two membranes became obvious (Fig. 5). However, after treatment with 3% (w/v) aqueous NaOH solution to remove mannan, protein, and lipid (see below) only one membrane is apparent at the bud scars (cf. Figs. 2 and 4).

The type of breakage resulting from disruption by glass beads, as applied to yeast cells, can be seen from the electron micrographs (Figs. 3 and 4d).

Chemical investigation of the cell wall

Elementary analysis. Total N, 2.1%, indicating a protein content of approx. 13%; total P, 0.31%.

Mineral content. When maintained at red heat in a platinum boat in a stream of clean air for 1 hr. 17.21 mg. yielded a white ash (0.55 mg., 3.21%).

Lipid content. The lipid was isolated by boiling the cell walls (17.39 mg.) in 95% (v/v) aqueous methanol (1 ml.) for 30 min. and subsequently extracting continuously with ether in a modified Soxhlet apparatus (Mitchell, 1951) at room temperature for 6 hr., the whole process being repeated three times. The ether was evaporated and the fat weighed directly. The yield was 1.48 mg. (8.5%). (Found: N, 0.1; P, 0.5%.) The fat thus appears to contain little phospholipid.

Acid hydrolysis. The preparation (8.40 mg.) was hydrolysed for 6 hr. with 1 ml. of 2N-HCl in a sealed tube at 98°; it dissolved completely to give a very light brown solution. This was evaporated to dryness at 20° under reduced pressure and dissolved in 0.1 ml. of water. The resultant solution was investigated on paper chromatograms. Only two sugars were apparent in the hydrolysate, namely mannose and glucose; no glucosamine could be detected. Although hydrolysis of protein was probably incomplete, the hydrolysate also showed the presence of glutamic acid, aspartic acid, serine, glycine, threonine and alanine with only very faint indications of histidine, leucine and the other basic amino-acids.

Water content. The cell wall material (10.8 mg.) was dried over P_2O_5 at 100° and 0.01 mm. Hg for 8 hr.; there was no loss in weight. Dried material (100 mg.) exposed to air at room temperature for 48 hr. increased in weight by 11.2%.

Polysaccharides of the cell wall

Isolation and investigation of the glucan component. The cell wall material (97.21 mg.) was digested with 3% (w/v) aqueous NaOH (2.0 ml.) for 6 hr. at 100°. The supernatant obtained after centrifuging was retained for examination of the dissolved mannan (see below). The insoluble residue was extracted consecutively with 3% NaOH at 100° for 6 hr.,

0.5N-acetic acid (2 ml.) at 75° for 6 hr. and finally washed with ethanol (2 ml.) and ether (2 ml.). The white solid was dried over P_2O_5 at 0.1 mm. Hg. Yield 27.9 mg. (28.8% of dry matter taken). (Found: N, 0.3; P, 0.0%.)

Hydrolysis of 10 mg. of this substance with 2N-HCl (1 ml.) in a sealed tube at 100° for 6 hr. gave a light-brown solution. This was freeze-dried and the resultant solid dissolved in 0.1 ml. of water. The chromatograms of this solution showed the presence of glucose only.

The isolated material contained 98% glucose when this was estimated by anthrone (Seifter, Dayston, Novic & Muntwyler, 1950). An equivalent amount of glucan isolated from whole yeast by the method of Bell & Northcote (1950) gave 97.4% glucose.

The substance isolated from the cell wall contained only glucose and was very insoluble in water, thus resembling the glucan isolated from whole yeast. It still retained in most cases the shape of the cell wall when examined under the microscope, and thus it constitutes at least part of a continuous membrane within the cell wall.

Isolation and investigation of the mannan component. The solution obtained in the above experiment was made just acid to methyl red with acetic acid, and the mannan was precipitated by 4 vol. of ethanol. The precipitate was centrifuged and separated from the supernatant, redissolved in water and reprecipitated. The white solid thus obtained was washed with ethanol and ether and dried in the normal manner. Yield, 30.1 mg. (31.0% of original dry matter). $[\alpha]_D^{20} + 87^\circ$ (l, 2; c, 1 in water). (Found: N, 1.3; P, 0.26%.)

This material (20 mg.) was hydrolysed as above for the glucan and gave a colourless solution. The hydrolysate was freeze-dried and dissolved in 1 ml. of water. Mannose phenylhydrazoné was prepared from this solution according to the method of Nowatnowna (1936). Yield 20.2 mg. (i.e. 60% mannose); m.p. 196° (uncorr.) not depressed on admixture of an authentic sample of mannose phenylhydrazoné (m.p. 196°). The cell wall mannan (30.0 mg.) was further purified by precipitation and subsequent decomposition of the copper compound (Haworth *et al.* 1937). Yield 25.0 mg. (N, 1.0; P, 0.2%). $[\alpha]_D^{20} + 88^\circ$ (l, 2; c, 1.0 in water). The hydrolysed mannan showed only mannose on a paper chromatogram. The mannan could also be extracted from the cell wall material by water at 100°, although the rate of extraction was considerably lower; 25 mg. gave 2.5 mg. of mannan after 6 hr. and a further 2.4 mg. after 12 hr. The mannan isolated in this manner had a relatively high nitrogen content (N, 2.5; P, 0.23%) and gave a positive biuret reaction.

Glycogen. No glycogen could be isolated from the cell wall preparation by means of 0.5N-acetic acid (75° for 12 hr.) nor could any glycogen be detected in the cell wall by staining with iodine and subsequent microscopical examination. The small particle fraction isolated by differential centrifugation of the broken yeast cell did, however, stain a dark brown with iodine.

PREPARATION B

Isolation of the cell wall material by digestion of the cells with sodium hydroxide solution

The whole yeast (12.62 g.) was dispersed in 15 ml. of 3% (w/v) aqueous NaOH and heated on a boiling-water bath for 6 hr. The brown solution was centrifuged and the residue redigested with a further 15 ml. of 3% NaOH for 3 hr.; it was then washed with water, ethanol and ether and dried in the normal manner. Yield, 0.13 g.

Microscopical examination of cell wall

This cell wall material showed an electron-dense substance scattered in granular and particulate masses throughout the walls (Fig. 6). Part of this material seemed to be glycogen as it could be seen under the optical microscope to stain brown with iodine. It could be dissolved away with 0.5*N*-acetic acid at 75° (see below). Apart from this the electron microscope examination of this preparation showed little difference from that of preparation *A*.

Chemical investigation of the cell wall

Elementary analysis. Total N, 0.7 %; total P, 0.07 %.

Acid hydrolysis. The hydrolysis was carried out in the same manner as for preparation *A*. Only glucose was apparent in the hydrolysate when this was examined on paper chromatograms.

Polysaccharides of the cell wall

Isolation and investigation of the glycogen component. The cell wall material (130 mg.) was digested with 0.5*N*-acetic acid (5 ml.) for 6 hr. at 75°. The residue was centrifuged and re-extracted with acetic acid for a further 6 hr. The acetic acid solutions were combined and evaporated to small bulk under reduced pressure when the glycogen was precipitated from solution by the addition of 6 vol. of ethanol. Yield of glycogen 25.1 mg. (19.3 % of the cell wall).

The glycogen could not be extracted with water from the cell wall, but after removal by dissolving in the dilute acetic acid and precipitation by ethanol the resultant material was very soluble in water to give a clear solution. This solution gave a deep red-brown colour with iodine.

Isolation and investigation of the glucan component. The residue obtained in the above experiment during the isolation of the glycogen corresponded to the glucan. It was washed with water, ethanol and ether and dried in the usual way. Yield, 93.3 mg. (71 % of the cell wall). This glucan corresponded exactly in appearance, glucose content and solubility, with that isolated from whole yeast and from the cell wall preparation *A*.

DISCUSSION

The literature contains very little information concerning the chemical and enzymic nature of the outer cell walls of plant or animal cells, and it was with this in mind that we attempted in the present research to isolate a part at least of the cell wall of yeast which might lend itself to further studies. A preparation has been obtained by mechanical breakage and differential centrifugation which is not contaminated by whole cells or cell debris. This preparation can be made with a fairly constant composition, free from substances present in other fractions of the cell and thus a clear-cut separation of a definite morphological structure has probably been achieved. The wall as isolated is made up of two or more membranes; two in fact can be clearly

seen by means of the electron microscope. It has been possible by relating the chemical investigation to the microscopical studies to show that one of these two apparent membranes is, in part, composed of the glucan polysaccharide since this polysaccharide can be isolated free from other material, still retaining the general shape of the whole cell and obviously constituting a complete membrane. The other membrane remains intact after removal of lipid; but when the protein and mannan are removed from the cell wall preparation by means of dilute sodium hydroxide solution it is no longer visible and thus this second membrane is made up in part of protein or mannan or both of these substances. The general analysis of the cell wall shows an approximate chemical composition of glucan 29%, mannan 31%, protein 13%, lipid (mainly neutral fat) 8.5 % and ash 3 % which accounts for over 84 % of the material. No other major constituent has been detected during the present investigation. Glycogen could only be obtained associated with the cell wall by using sodium hydroxide as a cytolytic agent. It is not present in the mechanically broken cell wall material, but appears among the fine particulate matter released by the breakage of the cell and the subsequent emptying of its contents. The glycogen present in the chemically prepared cell walls was seen to be present as scattered granules and it is thought that although it is probably closely associated with the cell wall in the living cell it does not form a structural part of the wall in the same way as the glucan and mannan.

The mannan can be extracted from the cell wall preparation *A* by means of hot water, and prepared in this manner it is found to be closely associated with protein which is extracted with it. This association in the cell wall had already been postulated by Garzuly-Janke (1940) from studies on the whole cell.

The observations carried out on the cell wall with the electron microscope and the phase-contrast microscope have shown clearly the existence on the cell surface of bud scars. These were first reported by Barton (1950) by microscopical examination of living and stained whole cells, although the observations were difficult and details of structure could not be obtained. The present work has shown that these scars are characterized by a circular thick edge slightly raised above the cell surface; the larger and presumably older cells carry a larger number of these scars. The edges of the scars are more resistant to chemical attack by dilute acid than is the general cell wall and this may indicate a difference in chemical composition. The existence of these scars must be important in relation to the process of budding and the behaviour of the cell wall as an osmotic boundary.

SUMMARY

1. A cell-wall fraction of yeast has been isolated after disintegrating the whole cells in a Mickle cell disintegrator and subsequently centrifuging.

2. The material isolated has been shown to be free of whole cells and cell debris.

3. A quantitative chemical analysis of the cell wall of yeast has shown it to be composed of protein, lipid and at least two polysaccharides, a mannan and a glucan. The glycogen associated with the cell wall preparation obtained by treatment of the whole cells with sodium hydroxide solution does not

function as a structural element and is not present in the fraction obtained by mechanical breakage.

4. The mannan is associated with a protein present in the cell wall.

5. The cell wall has been examined by the optical and electron microscopes and has been shown to consist of at least two membranes, one of which is made up in part of the glucan component.

6. The existence of bud scars on the cell wall has been confirmed and some details of their structure observed.

We wish to thank Dr V. E. Cosslett and Dr D. J. Bell for their advice and encouragement during this investigation.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. Intact yeast cell showing budding.

Fig. 2. Group of cell walls (preparation A) showing bud scars and peeling of two apparent membranes at the scars.

Fig. 3. Cell wall (preparation A) showing junction between two budding cells together with other bud scars.

Fig. 5. Fragment of cell wall (preparation A) after extraction of lipid showing two distinct membranes.

Fig. 6. Cell wall prepared by cytolysing whole cells with 3% NaOH (preparation B) showing the granular appearance of the glycogen.

PLATE 2

Fig. 4. Cell wall (preparation A) treated with 3% NaOH.
 (a) Cell membrane showing numerous bud scars and disappearance of double membrane apparent in Fig. 2.
 (b), (c) and (d) Showing, in particular, details of structure of cell membrane at the budding point; note thickening and raised appearance of the membrane.

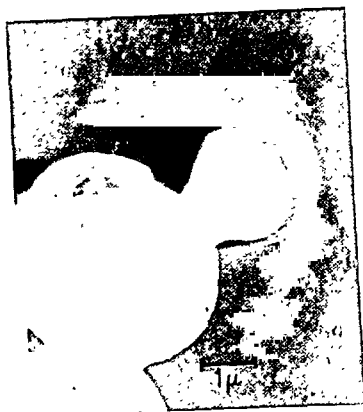


Fig. 1.



Fig. 2.

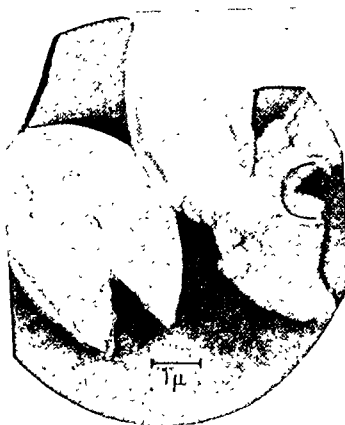


Fig. 3.



Fig. 5.

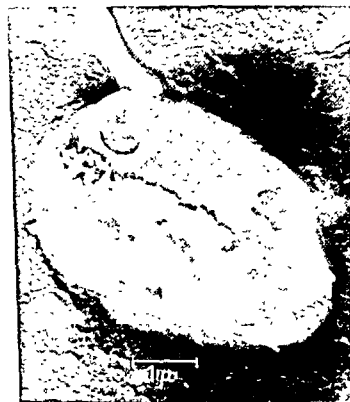


Fig. 6.

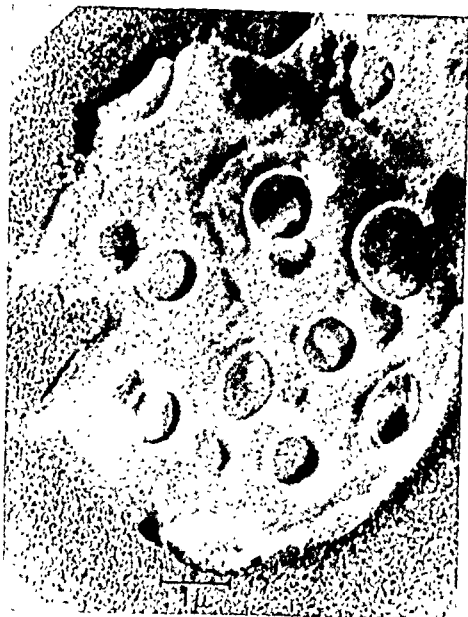


Fig. 4 a.



Fig. 4 b.

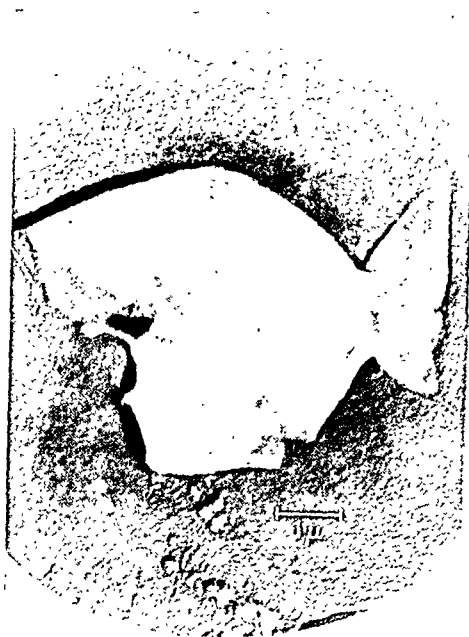


Fig. 4 c.



Fig. 4 d.

The Estimation of Vitamin E

1. SEPARATION OF TOCOPHEROL MIXTURES OCCURRING IN NATURAL PRODUCTS BY PAPER CHROMATOGRAPHY

By F. BROWN

The Hannah Dairy Research Institute, Kirkhill, Ayr

(Received 30 August 1951)

The determination of the tocopherol content of biological materials by the ferric chloride-2:2'-dipyridyl method (Emmerie & Engel, 1938) gives results which are of limited value for two reasons. First, the biological activities of the α -, β -, γ - and δ -tocopherols are in the approximate ratio 100:30:20:1, depending on the biological test used, whereas all four tocopherols give the same value by the chemical method. Secondly, non-tocopherol substances which also give a red colour with the Emmerie-Engel reagents are often difficult to separate from the tocopherols and it is not always possible to be certain that the interfering impurities have been removed from the solutions which are finally tested (Baxter, Lehman, Hove, Quaife, Weisler & Stern, 1947).

Differential analysis of the tocopherols can be partly achieved by coupling the γ - and δ -compounds with diazotized *o*-dianisidine (Weisler, Robeson & Baxter, 1947); the α - and β -tocopherols do not couple with this reagent. Since β -tocopherol has been found in wheat products only, values for the total and γ -plus δ -tocopherol contents of most materials can be obtained by examining for (a) total tocopherols by the Emmerie-Engel method, and (b) γ -plus δ -tocopherols by coupling with diazotized *o*-dianisidine. The value for α -tocopherol can be obtained by subtracting (b) from (a).

The desirability of a method which would allow a clear-cut separation of the tocopherols led Emmerie (1949) to attempt the resolution of the tocopherols by chromatographing on Floridin XS Earth or alumina. Although the α - and γ -tocopherols could be separated under strictly controlled conditions, mixtures of the α - and β -tocopherols could not be resolved into the separate components.

Since paper chromatography has found application in numerous problems involving the separation of closely related compounds, attempts have been made in the present work to extend the method to the analysis of tocopherol mixtures made up in the laboratory. Tocopherol mixtures occurring in natural products have also been examined in order to test the utility of the method in distinguishing non-tocopherol reducing substances from authentic tocopherols.

EXPERIMENTAL

Chromatography

Both ascending (Williams & Kirby, 1948) and descending (Consden, Gordon & Martin, 1944) methods were used. Whatman no. 1 filter paper was coated with Vaseline (Winteringham, Harrison & Bridges, 1950) by dipping it in a 2.5% (w/v) solution of Vaseline in ether and then allowing the solvent to evaporate from it in the air. Storage of the coated papers for as long as 2 months before use did not alter their properties. The tocopherols were applied to the papers by means of a calibrated pipette delivering 6.3 μ l. Alcoholic solutions of the tocopherols were used to avoid dissolving the Vaseline at the points of application. Various concentrations of methanol, ethanol and acetone in water were each tested for use as the mobile phase and 75% ethanol (v/v) was finally selected as giving the best separations.

Detection of the tocopherols

The developed chromatograms were dried for 15 min. at room temperature and then sprayed with 2:2'-dipyridyl (0.25%, w/v) in ethanol followed by ferric chloride (0.1%, w/v) in ethanol. Bright-red spots were formed on a white background. The background gradually assumes a pink colour so the positions of the spots must be marked fairly soon after spraying. In order to obtain a permanent record, methanolic silver nitrate (1.7 g. AgNO₃ in 50 ml. water, 33 ml. methanol and 17 ml. aqueous NH₃ sp.gr. 0.880) can be used. The dark brown spots appear about 1 min. after spraying, the one corresponding to the α -form appearing first and that corresponding to the δ -form last. Spraying with 2% (w/v) Na₂CO₃ followed by diazotized *o*-dianisidine solution (prepared according to the method of Weisler *et al.* 1947) was used for detecting the γ - and δ -tocopherols. The γ -compound gives a dark-purple spot, whereas the δ -compound gives a red spot readily distinguishable from the γ -spot. Under these conditions the α - and β -compounds do not give spots.

Separation of synthetic mixtures of the tocopherols

Mixtures of racemic α -, β - and δ -tocopherols were readily separated using 75% (v/v) aqueous ethanol as the mobile phase and Vaseline-coated filter papers as the stationary phase; the β - and γ -isomers could not be separated. The *R_F* values of the tocopherols were: α , 0.50; β , 0.72; γ , 0.72; δ , 0.84. These values were the same whether the tocopherols were used alone or mixed with other tocopherols.

Inability to separate the β - and γ -isomers is not a serious

drawback in examining natural products because the β -compound occurs only in wheat products. In addition, if a spot is obtained at $R_F=0.72$ using the 2:2'-dipyridyl-ferric chloride reagents, the presence of γ can be ascertained by using the *o*-dianisidine spray.

As little as 3 μ g. of any of the tocopherols can be readily detected with the dipyridyl-ferric chloride reagents and even less of the γ - and δ -compounds can be detected with the sodium carbonate-*o*-dianisidine spray.

Extraction of the tocopherols from biological material

Methods for the extraction of tocopherols from natural products are largely based on methods for fat extraction (Quaife & Harris, 1948; Quaife & Dju, 1949). Lipids and sterols interfered with the running of the tocopherols on the chromatograms and were consequently removed. The lipids were hydrolysed in the presence of pyrogallol (Tošić & Moore, 1945) to prevent loss of tocopherols by oxidation, and the unsaponifiable matter freed from sterols by crystallizing from methanol solution at -15° . Carotene in large amounts also caused interference on the chromatograms; consequently the carotenoids were removed by absorbing them from benzene solution on Floridin Earth according to the method of Emmerie & Engel (1939). The Floridin Earth 'specially prepared for use in Emmerie's test for tocopherols' as supplied by British Drug Houses Ltd. was found to cause some destruction of the tocopherols. Glavind, Kjølhede & Prange (1942) have also reported that in the passage of tocopherols through earths of high surface activity a considerable amount of the tocopherols is oxidized. They prevented the oxidation by boiling the earth (1.5–2.0 g.) with SnCl_2 (0.25 g.) and 10N-HCl (5–8 ml.) before using it. This procedure has been adopted in the present work, the heated mixture being introduced into an adsorption tube and washed free from acid with ethanol (5 ml.) followed by benzene (5 \times 5 ml. portions). Using this method of preparation quantitative recoveries of the tocopherols have been obtained using as little as 10 μ g. of tocopherols. Some, if not all, of the destruction of the tocopherols when using B.D.H. Floridin Earth was due to the presence of iron in the earth.

Examination of tocopherol mixtures present in natural oils

Vegetable oils are among the richest natural sources of the tocopherols, and consequently afford excellent starting materials for testing the method described. The oils were hydrolysed (Tošić & Moore, 1945) and the unsaponifiable fraction in benzene passed through a prepared column (30 \times 13 mm.) of Floridin Earth (see above). For each oil the filtrate was evaporated under reduced pressure and the residue dissolved in warm methanol. After removing the sterols by crystallizing at -15° , the mother liquors were applied to the starting line of the paper. The tocopherols found in several oils are shown in Table 1.

Treatment of the chromatogram of the wheat-germ oil with the 2:2'-dipyridyl-ferric chloride reagents gave, in addition to spots at the positions corresponding to the α - and β -tocopherols, a distinct spot at $R_F=0.93$. Ammoniacal silver nitrate was reduced by the substance, but a spot was not obtained with the *o*-dianisidine reagent. The identity of this substance was not ascertained. The spot at $R_F=0.72$ was β - and not γ -tocopherol because spraying with sodium

carbonate and diazotized *o*-dianisidine failed to give a spot. None of the other oils examined gave an 'apparent tocopherol' spot in this way, indicating that all the interfering substances had been removed prior to chromatography.

Table 1. *Tocopherols found to be present in various natural products*

Substance	Tocopherol			
	α	β	γ	δ
Arachis oil	+	.	+	.
Cottonseed oil	+	.	+	.
Soya-bean oil	+	.	+	+
Wheat-germ oil	+	+	.	.
Cocksfoot grass	+	.	.	.
Fescue grass	+	.	.	.
Timothy grass	+	.	.	.
Cows' blood	+	.	.	.
Cows' milk	+	.	.	.

In the soya-bean oil chromatogram three spots with R_F values 0.50, 0.72 and 0.84 were obtained with the Emmerie-Engel reagents. The presence of γ - and δ -tocopherols was confirmed by means of the *o*-dianisidine spray.

Tocopherols occurring in other natural products

The importance of the tocopherols in the nutrition of farm animals is becoming increasingly apparent. Consequently, three different species of grass, timothy (*Phleum pratense*), cocksfoot (*Dactylis glomerata*) and fescue (*Festuca pratensis*), have been examined to determine which tocopherols they contain. The grasses were extracted with ethanol in a Soxhlet apparatus and the ethanol solution extracted with light petroleum (Quaife & Harris, 1948). The unsaponifiable fractions were examined in the same way as those from the oils. Only α -tocopherol was found in the grasses (Table 1) but there is present in cocksfoot a substance which gives a purple colour with Na_2CO_3 and diazotized *o*-dianisidine. This unidentified substance moved only a short distance from the starting line on the chromatogram (R_F approx. 0.05).

The blood and milk of cows feeding on the three grasses contained α -tocopherol only. Because of the relatively low levels of tocopherols in cows' blood and milk (about 800 and 150 μ g./100 ml. respectively), even when the dietary intake is 10 g. or more daily (Cabell & Ellis, 1942), about 10 ml. of blood serum and 50 ml. of milk are necessary for a satisfactory analysis to be made.

DISCUSSION

The Emmerie-Engel method for determining tocopherols depends on the reduction of alcoholic ferric chloride followed by reaction of the ferrous salt with 2:2'-dipyridyl to give a red ferrous-dipyridyl complex. Since other substances likely to be present in the fat-soluble fraction of natural products can also reduce ferric to ferrous iron, these interfering substances must be removed in order to obtain the true tocopherol content. Even then comparison with biological assays is difficult because of the different biological potencies of the tocopherols, and the fact that the Emmerie-Engel reaction does not distinguish between the different compounds.

The method described in the present work allows the purity of the solution under test for tocopherols to be assessed and also enables the separation of the commonly occurring α -, γ - and δ -tocopherols to be achieved. Applications of the method to the examination of several oils indicate the usefulness of the method. For example, the presence of δ -tocopherol in soya-bean oil is readily ascertained, whereas its existence escaped detection by conventional methods for many years. In addition, the substance in cocksfoot grass which gives a colour with diazotized *o*-dianisidine is shown to be different from any of the known tocopherols in its behaviour on the chromatogram. Similarly, a non-tocopherol ferric-reducing substance which is not removed by the saponification, adsorption and crystallization processes is present in wheat-germ oil.

Although the method will probably not be readily applicable to routine analysis, its value for reliably

detecting the presence of naturally occurring tocopherols is apparent. Application of the method to the quantitative analysis of tocopherol mixtures is being undertaken and will be the subject of a further communication.

SUMMARY

1. A method is described for the detection of α -, β -, γ - and δ -tocopherols by filter-paper chromatography.

2. The method has been used for study of the tocopherols in various vegetable oils and grasses and also in blood and milk.

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Flavin-adenine Dinucleotide and Diaphorase in Resting and Germinated Spores, and Vegetative Cells of *Bacillus subtilis* and *Bacillus megatherium*

By ROSEMARY E. J. SPENCER AND JOAN F. POWELL

Microbiological Research Department (Ministry of Supply), Porton, Wiltshire

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There are conflicting reports on the respiration rate of resting bacterial spores, but it is generally agreed that it is lower and less sensitive to cyanide than that of vegetative cells (Tarr, 1933; Keilin & Hartree, 1947). Keilin & Hartree (1949) have also found that although the spores of *Bacillus subtilis* National Collection of Type Cultures 85 (N.C.T.C.) contain considerable amounts of unidentified haematin compounds, their cytochrome content is only 6% of that of vegetative cells, and more recently Chaix & Roncoli (1950) have observed development from an atypical to a classical cytochrome spectrum during the growth of several strains of *B. subtilis*. These findings suggest that

bacterial spores differ from vegetative cells in possessing an alternative to the cytochrome-cytochrome oxidase system, possibly in the form of a flavoprotein reacting with oxygen, either directly or through an unidentified haematin catalyst (Slater, 1949*a, b*) and that this flavoprotein system may be replaced by the cytochrome system as growth proceeds.

It is interesting to note that respiration, relatively insensitive to cyanide, has also been demonstrated in resting spores of *Neurospora crassa* (Goddard & Smith, 1938) and in unfertilized *Arbacia* eggs (Korri, 1939). The latter were shown to contain haematin compounds but no cytochrome (Ball & Meyerhof,

1940), flavin-adenine dinucleotide (Krahl, Keltch & Clowes, 1940), and a substance resembling cytochrome oxidase (Krahl, Keltch, Neubeck & Clowes, 1941). A direct connexion between decreased cyanide-sensitivity and flavoprotein synthesis in yeast was reported by Pett (1935) who found that the flavoprotein content of this organism was doubled during growth in a medium containing cyanide. In this medium respiration was reduced, although fermentation remained at a normal level.

It seemed of interest, therefore, to determine the relative amounts of flavin-adenine dinucleotide in resting spores, germinated spores (Hills, 1950; Powell, 1950) and fully developed vegetative cells of *B. subtilis* and *Bacillus megatherium*, and to attempt to demonstrate the presence of enzymes containing flavin-adenine dinucleotide as their prosthetic group.

ORGANISMS AND METHODS

Organisms

Spores of the laboratory strain of *B. subtilis* used by Hills (1950) and of *B. subtilis* N.C.T.C. 85 were grown on CCY agar (Gladstone & Fildes, 1940) at 37°. They were reaped after a minimum of 3 days, washed five times with distilled water and stored at room temperature. Vegetative cells of the laboratory strain were grown on meat extract-peptone agar (Tarr, 1933) containing 1% Lab-Lemco, 1% peptone, and 0.5% NaCl, for 16 hr. at 28°. The cells were reaped and washed twice with large volumes of saline. No spores were visible in a stained film.

Spores of the laboratory strain require L-alanine specifically for optimal germination (Hills, 1950). During 30–60 min. incubation at 37° in a medium containing 5 mM-L-alanine and 50 mM-glucose buffered at pH 7.3 with 33 mM-phosphate, 90% of the spores in a 10⁹/ml. suspension lose their heat resistance, becoming at the same time less refractile and more permeable to stains (Powell, 1950). Germinated forms for the present investigation were obtained in this way and were centrifuged and made up to a suitable concentration with distilled water.

A chemically defined medium in which spores of *B. subtilis* N.C.T.C. 85 will germinate has not yet been found, so that comparable results are only available for the resting spores of this organism.

Spores of a freshly isolated strain of *B. megatherium* were grown on meat extract-peptone agar (Tarr, 1933) containing 1% peptone, 0.2% Lab-Lemco, 0.5% NaCl and 0.2% glucose. They were reaped after 2 days at 37°, washed three times with distilled water, heated to 60° for 30 min. and finally washed twice more. It was found necessary to ice-cool suspensions of these spores until they had been washed several times, as inadequately washed suspensions germinated completely at room temperature (Powell, 1951). Vegetative cells were grown on CCY agar, reaped after 16 hr. at 28° and washed twice with saline. No spores were detectable in a stained film.

Germinated spores were obtained by incubating 10⁹/ml. suspensions at 37° for 60 min. in 50 mM-glucose and

33 mM-phosphate buffer at pH 7.3. During this time about 90% of the spores showed the changes associated with germination (Powell, 1951) described above.

Methods

Determination of flavin-adenine dinucleotide by combination with the protein of D-amino-acid oxidase. The method described by Warburg & Christian (1938) was used. To facilitate extraction of flavin-adenine dinucleotide (FAD) the spores, germinated spores or vegetative cells were broken in a Mickle (1948) tissue disintegrator. A maximum of 12 ml. of a suspension containing 10–15 mg./ml. dry weight were shaken with 8 g. Ballotini beads, size 12, for 45–50 min., a drop of tributyl citrate being added to prevent foaming. After this treatment, very few intact cells could be seen in a stained film. Oxygen uptake was measured at 37° in Warburg vessels containing the broken cell material, an appropriate D-amino-acid, and an excess of D-amino-acid oxidase protein, in an atmosphere of oxygen. The system was buffered at pH 7.3 rather than at the optimum pH 8.8, since Krebs (1935) and Ochoa & Rossiter (1939) report a rapid fall in activity of the enzyme under alkaline conditions. Controls were set up in which the cell material was replaced by a crude preparation of FAD. Total FAD was determined on a portion of the broken cell suspension which had been heated to 100° and held at 75–80° for 10 min. in order to split the FAD from its enzyme proteins. Free FAD was determined on unheated samples.

Preparation of D-amino-acid oxidase protein. The apoenzyme of D-amino-acid oxidase was prepared from pig kidneys by a modification of the method of Negelein & Brömel (1939). Kidney cortex was minced and immediately freeze-dried. The freeze-dried material was then finely powdered and treated twice with large volumes of acetone. The acetone was removed by straining the tissue in a muslin bag, and then passing a stream of dry air through the powder for several hours. Although the freeze-dried material had quite high D-amino-acid oxidase activity before the acetone treatment, further purification omitting this step resulted in an entirely inactive preparation. The enzyme was extracted from the dried powder with 10 mM-phosphate buffer at pH 7.3 instead of the pyrophosphate used by Negelein & Brömel (1939), and the extract was treated as described in steps 1–5 of the above method. The final precipitate was freeze-dried and kept in a desiccator.

Preparation of flavin-adenine dinucleotide. The prosthetic group of D-amino-acid oxidase was prepared from baker's yeast (United Yeast Co.) according to the method of Warburg & Christian (1938) with a modification in the final stages suggested by the work of Straub (1938). The method was followed as far as the first precipitation of FAD with barium and the removal of the barium as barium sulphate. At this stage, the FAD was precipitated with a large volume of acetone (Straub, 1938), centrifuged, redissolved in a small volume of water and freeze-dried.

Absorption maxima for this preparation occurred at 260, 375 and 450 mμ. (Unicam spectrophotometer). Molecular extinctions at 375 and 450 mμ. were compared with those of the pure barium salt (Warburg & Christian, 1938) and indicated that the crude preparation contained not more than 10% (Table 1) of flavin-adenine dinucleotide, and probably rather less, since absorption by any free riboflavin present as an impurity was not taken into account.

An accurate estimate of the purity of this FAD preparation was obtained by comparison of its activity as the prosthetic group of D-amino-acid oxidase, with that of a sample of the barium salt of FAD (Warburg & Christian, 1938) containing only 13% of water. The conditions were the same as those used in the estimation of FAD in cell material. The weights of the two preparations were accurately determined on a microbalance and checked by measuring the extinctions of the solutions in the Unicam spectrophotometer at 450 m μ .

The molecular extinction coefficient ϵ is the optical density of a solution of concentration 1 g.mol./l. and path length 1 cm. Warburg & Christian (1938) measure the absorption coefficient, μ , which is the natural logarithm of the reciprocal transmission

$$\epsilon = \mu / (2.3026 \times 10^3).$$

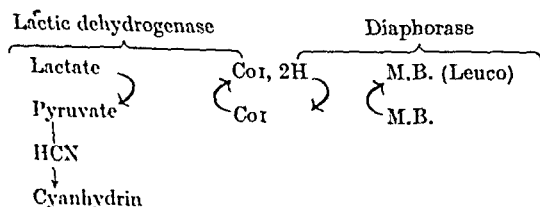
Table 1. Spectrophotometric absorption data for flavin-adenine dinucleotide preparation

Wavelength (m μ)	260	375	450
Concn. of solution of crude FAD in μ g. total solid/ml.	89	445	445
Extinction coefficient of crude FAD solution	1.3	0.50	0.535
Molecular extinction of crude FAD solution	1.34×10^4	0.103×10^4	0.111×10^4
Molecular extinction of pure barium salt (Warburg & Christian, 1938)	3.64×10^4	0.895×10^4	1.13×10^4
Percentage FAD in crude preparation	36.8	11.5	9.85

Detection of flavoproteins. L-Amino-acid oxidase, D-amino-acid oxidase and xanthine oxidase were assayed by measuring O₂ uptake, in an atmosphere of O₂, in the presence of the specific substrate.

For the detection of amino-acid oxidases, the system contained 16 mM-amino-acid, 40 mM-phosphate buffer at pH 7.3 and 10–20 mg. dry weight of cell material. Synthetic DL-alanine, valine, phenylalanine, norleucine and methionine were used. L-Leucine and L-proline were also tested at a concentration of 8 mM. To detect xanthine oxidase 3.3 mM-xanthine was substituted for the amino-acid.

The system used to detect diaphorase was that of Straub (1939; 1940) and Sumner & Krishnan (1948). It contained 0.2 ml. 10% sodium lactate, 0.5 ml. lactic dehydrogenase solution, 1 ml. 2% (w/v) HCN, 20–40 μ g. coenzyme I (CoI), 1 ml. diaphorase solution, 0.1 ml. 0.05% (w/v) methylene blue, and 0.5 ml. 200 mM-phosphate buffer, pH 7.3. The reaction proceeds anaerobically thus:



diaphorase activity being indicated by decolorization of the methylene blue.

The method of Friedemann & Hollander (1942) was used in this test, because a qualitative result only was required. The results obtained cannot be regarded as quantitative owing to the presence of dissolved oxygen in the medium. An equal volume of 2% (w/v) melted agar, cooled to 45°, was added to the complete system contained in an ice-cooled test-tube. The contents of the tube were thoroughly mixed and when the agar had solidified (1 min.) the tube

was transferred to a water bath maintained at 38°. The time needed for 90% reduction of the methylene blue was noted. Broken suspensions of resting and germinated spores and of vegetative cells, previously analysed for FAD were substituted for diaphorase and their activities compared. Some cell suspensions were also tested in the presence of added diaphorase, and of larger amounts of CoI (1 mg.). In a few experiments with broken spores the location of the enzyme activity was determined by testing the supernatant liquid and the cell debris separately after centrifuging.

Preparation of lactic dehydrogenase. The enzyme was prepared from ox heart by a modification of Straub's (1940) method. The heart muscle was minced, and the enzyme extracted with ice-cold water and precipitated by 60% saturation with ammonium sulphate. After centrifuging in the cold, the precipitate was dissolved in 10 mM-phosphate

buffer, pH 7.3, and recentrifuged to remove insoluble material. A second precipitation at 60% saturation with ammonium sulphate gave a preparation which, when dissolved in a small volume of phosphate buffer, retained its activity during 2 months' storage at 4°. The enzyme solution was subsequently freeze-dried without loss of activity.

Preparation of soluble diaphorase. The method of Straub (1939) was used, except that in the initial treatment a Waring blender was employed to break up the heart muscle. The method was then followed to the stage in which the diaphorase was brought into solution. The activity of this solution was not diminished during 2 months' storage at 4° or by freeze drying.

Spore respiration. Oxygen uptake of spores was measured in Warburg manometers in 33 mM-phosphate buffer pH 7.3 at 37°, with and without the addition of 50 mM-glucose. In determinations of the cyanide-sensitivity of the respiration, steady overall concentrations of cyanide were maintained by the use of KOH-KCN mixtures in the centre well (Potter, 1948).

Chemicals. Coenzyme I (cozymase) was a commercial preparation supplied by L. Light and Co. Ltd., who also supplied DL-alanine, DL-valine, DL-phenylalanine, and DL-norleucine, and L-leucine and L-proline. L-Alanine was supplied by Roche Products Ltd. HCN 2% (w/v) was prepared freshly by neutralizing a KCN solution with H₂SO₄.

RESULTS

Flavin-adenine dinucleotide content of cells

A system containing 3 mg. D-amino-acid oxidase protein, 33 mM-DL-methionine, 2.7 μ g. crude FAD and 75 mM-phosphate buffer (pH 7.3) in a total volume of 2.5 ml. gave an oxygen uptake of 53 μ l./10 min. The rate of oxygen uptake was directly proportional to the amount of FAD added, provided

that the apoenzyme was in excess. With methionine as substrate the rate was four times as great as with DL-alanine. This activity ratio is twice that found by Krebs (1935) and is probably due to the presence of inhibitory substances in the synthetic DL-alanine, since another batch of this amino-acid inhibited the enzyme completely. Methionine was therefore generally used as substrate.

When disintegrated cells were substituted for crude FAD in the above system an oxygen uptake was recorded proportional to the amount of cell material added. Thus the relative amounts of free and combined FAD in spores, germinated spores and vegetative cells could be estimated. Compared with the pure barium salt of FAD, the preparation used as a standard contained 76.8 ± 3.0 mg. active FAD/g. The values given in Table 3 for the total FAD content of the cells examined are calculated from this estimation of the purity of the standard. The standard error quoted is that of the mean of two experiments from each of which quadruplicate results for both preparations were obtained.

The results of a typical experiment are summarized in Table 2. It was found that, in the absence of

the substrate, or FAD, or the apoenzyme, the system was inactive. No oxygen uptake was recorded when cell material was substituted for FAD, unless both substrate and enzyme protein were present.

In a duplicated experiment, 40 μ g. of FAD preparation were added to 10 ml. of spore suspension, the total and free dinucleotide activity then being measured in the usual way. The results indicated that none of the added FAD was destroyed during cell breakdown, but that when the total dinucleotide activity was determined after heating, there was a 30% fall in activity of the added material. On the other hand, when a broken spore suspension was heated for 30 min. at 75–80°, no fall in total dinucleotide activity was detected. It therefore seems probable that the partially purified FAD is more thermolabile than the FAD in the spore.

Experiments in which broken spore suspensions were centrifuged and the supernatant liquid substituted in the test system, showed that FAD activity was not associated with the cell debris. The addition of tri-butyl citrate, used to prevent foaming during cell breakdown, was found to have no effect on the oxygen uptake of the D-amino-acid oxidase system.

Table 2. Protocol of experiment to assay flavin-adenine dinucleotide content of *Bacillus subtilis* spores

	Controls (ml.)				Unheated spores (ml.)				Spores heated to 100° (ml.)			
	1	2	3	4	5	6	7	8	9	10	11	12
Manometer ...												
Water	1.25	1.25	1.5	1.0	0.5	0.75	0.25	0.75	0.5	0.75	0.25	0.75
Phosphate buffer, 200 mM, pH 7.3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine, 163 mM (in side arm)	Nil	0.25	0.25	0.25	Nil	0.25	0.25	0.25	Nil	0.25	0.25	0.25
Flavin-adenine dinucleotide, 8 μ g. crude preparation/ml.	0.25	Nil	0.25	0.25	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
D-Amino-acid oxidase protein, 6 mg./ml.	0.5	0.5	Nil	0.5	0.5	Nil	0.5	0.5	0.5	Nil	0.5	0.5
Broken suspension of <i>B. subtilis</i> spores	Nil	Nil	Nil	Nil	1.0	1.0	1.0	0.5	1.0	1.0	1.0	0.5
5.5 mg./ml. (dry wt.)												
20% (w/v) KOH (in centre well)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Oxygen uptake (μ l./10 min.)	0	0	0	40	0	0	21	10.5	0	0	41	20

Table 3. Flavin-adenine dinucleotide content of bacterial cells

(The content was estimated by measurement of the O₂ uptake of the unheated and heated suspension (=free and bound FAD) in presence of D-amino-acid oxidase apoenzyme + methionine. 1 ml. O₂/10 min. = 3.56 μ g. FAD.)

	No. of experiments	Dry wt. of cells (mg.)	Spore count	Mean O ₂ uptake of unheated suspension. Free FAD (μ l./10 min.)	Mean O ₂ uptake of heated suspension. Total FAD (μ l./10 min.)	Percentage combined FAD	Total FAD (μ g./g. dry wt.)
<i>B. subtilis</i> (lab. strain):							
Spores	5	5.56	10 ¹⁰	22.2	42.2	48	29.4
Germinated spores	4	2.2	10 ¹⁰	20.25	39.6	49	—
		(approx.)					
Vegetative cells	4	1.8	—	3.0	27.5	80	123.0
<i>B. megatherium</i> :							
Spores	11	7.76	10 ¹⁰	9.3	59.0	84	29.7
Germinated spores	5	—	10 ¹⁰	9.2	59.2	85	—
Vegetative cells	4	2.07	—	2.0	40.0	90	98.0
<i>B. subtilis</i> N.C.T.C. 85:							
Spores	3	7.35	2.2 × 10 ¹⁰	52	54.5	5	28.9

The figures for FAD content of resting spores of two strains of *B. subtilis* and one strain of *B. megatherium* are given in Table 3. Although there were great differences in the ratio of free to combined FAD in the three organisms, the total amounts were remarkably similar.

Vegetative cells contained considerably greater amounts (3-4 times) of FAD. Comparisons were made on a basis of dry weight, which was determined by heating continuously at 104° for at least 48 hr. In germinated spores of the laboratory strain of *B. subtilis* and *B. megatherium*, both the total FAD and the ratio of free to combined FAD were the same as in resting spores on the basis of spore number. In this case, dry weight was not considered a satisfactory basis for comparison since germinated spores of *B. subtilis* were found to have a dry weight less than half that of the same number of resting spores, although no cell division had occurred. An explanation is at present being sought for this result. Slight inaccuracies must arise in this determination since after centrifuging the germinated spore suspension small amounts of medium still remain associated with the residue. Washing would have removed these, but might have introduced further errors, for example, by extracting soluble material from the germinated spore. The dry weight of germinated spores of *B. megatherium* was not determined.

Occurrence of flavoproteins

L-Amino-acid oxidase and D-amino-acid oxidase. No oxygen uptake generally occurred with resting spore suspensions of the laboratory strains of *B. megatherium* and *B. subtilis* in the presence of any of the amino-acids tested. In some cases in which a small oxygen uptake was recorded, it could be attributed to germination during the course of the experiment. Germinated spores possessed a low amino-acid oxidase activity (1 μ l./hr./mg. dry weight) which was completely lost when the suspension was broken up.

Xanthine oxidase. Negligible rates of oxygen uptake were observed in the cells tested with xanthine as substrate. Vegetative cells of *B. subtilis* gave the highest oxygen uptake, which was only about 1 μ l./hr./mg. dry weight.

Diaphorase. An enzyme similar in its action to the diaphorase of heart muscle was present in all cells of the three organisms studied. The enzyme remained in the supernatant liquid after the broken cell suspension had been centrifuged at 1100 \times g for 20 min. and was completely inactivated by heating for 5 min. at 100°. The diaphorase-like enzyme in resting and germinated spores of *B. megatherium* required the addition of much larger amounts of CoI for its demonstration than did that in the two

strains of *B. subtilis*. It was later found that the activity of the heart diaphorase preparation was completely inhibited by broken spores of *B. megatherium*. The inhibition could be prevented by the addition of larger amounts (1 mg.) of CoI, but not by excess FAD. It seems that the spores of *B. megatherium* contain a substance which either removes, or competes with, CoI, under the experimental conditions. The inhibitor was associated with the cell debris and was stable during 10 min. at 100°. No such substance was present in the spores of *B. subtilis*, and moreover the action of the diaphorase-like enzyme which they contain was only slightly inhibited by addition of broken *B. megatherium* spores. This effect could not be accounted for by the presence of relatively large amounts of CoI in *B. subtilis* spores, since they were unable to reduce methylene blue when added to the dehydrogenase system in the absence of added CoI.

It may be significant that the times for reduction of methylene blue by the various cells were roughly in inverse proportion to the amount of bound FAD contained in these cells.

Oxygen uptake of spores

We have attempted to measure the oxygen uptake of resting spores of the laboratory strain of *B. subtilis* in phosphate buffer only. None could be detected using a micro-Warburg apparatus (Barker, 1949) containing up to 30 mg. dry weight of a ten-times washed suspension. A measurable, but variable, oxygen uptake occurred in the presence of 50 mM-glucose. This could be reduced by heating at 60° for 15 min., the Q_{O_2} over an experimental period of 2.5 hr. then being 0.42 compared with Q_{O_2} of 2.1 over the same period reported by Keilin & Hartree (1947) for spores of *B. subtilis* N.C.T.C. 85. The oxygen uptake of spores of the laboratory strain in 50 mM-glucose was found to increase 200-fold after 30 min. incubation in 5 mM-L-alanine. This high oxygen uptake, associated with germinated spores, was found to be considerably less sensitive to cyanide than that of vegetative cells. Thus 0.1 mM-HCN caused 54 % inhibition of vegetative cell respiration and 24 % inhibition of germinated spore respiration. Similar results were obtained with germinated spores and vegetative cells of *B. megatherium*. Here, 0.2 mM-HCN gave 70 % inhibition of vegetative cell respiration and 40 % inhibition of germinated spore respiration. Corresponding figures for 0.4 mM-HCN were 79 and 58 % respectively. These results imply that the oxygen uptake of resting spores may be even lower than that hitherto recorded, and that the cyanide-insensitive respiration previously considered to be a property of resting spores may be in fact due to a small proportion of germinated forms present in the suspensions.

DISCUSSION

Flavin-adenine dinucleotide and an enzyme closely resembling diaphorase in function have been found in all the resting spores, germinated spores and vegetative cells tested. Although the total amounts of flavin-adenine dinucleotide in spores of the three organisms investigated are very similar, the ratios of free to combined dinucleotide differ considerably. Diaphorase activity appears to run roughly parallel with the proportion of bound dinucleotide. These findings may not, however, represent the state in the intact spore, since it is possible that flavin-adenine dinucleotide is split from its apoenzyme to varying degrees in different organisms during cell breakdown.

During germination, i.e. the primary change involving loss of heat resistance of spores, no alteration could be detected in the total, or in the ratio of free to combined, flavin-adenine dinucleotide, when the comparison was made on the basis of spore number. Vegetative cells contained three to four times as much flavin-adenine dinucleotide per unit of dry weight and 80–90% of this was in the combined state. They also had a much higher diaphorase activity than spores. A comparison between spores and vegetative cells based on dry weight must, however, be made with some caution, since the spore coat contributes an unknown proportion of the dry weight. Even with this reservation it does not seem that development from spore to vegetative cells is accompanied by a fall in flavin-adenine dinucleotide content or diaphorase activity. In view of the findings of Curran, Dewar, Gordon and Green (1939), indicating that xanthine oxidase can replace diaphorase in the dehydrogenase-methylene blue system, it is important to note that negligible xanthine oxidase activity was detected in the organisms tested.

It is significant that the respiration rate of spores of *B. subtilis* N.C.T.C. 85 was reported respectively by Cook (1931), Tarr (1933) and Keilin & Hartree (1947) as 90, 40 and 6% of that of vegetative cells. It seems probable that the spore suspensions used contained germinated forms not easily characterized except by their lack of heat resistance and slight difference in staining properties (Powell, 1950). For instance, in the spore suspensions employed by Tarr (1933) fully developed vegetative cells were observed after 2 hr. incubation, so that, in this case at least, germination must have taken place at a considerably earlier stage in the experiments. Comparative insensitivity to cyanide cannot be taken as a criterion of resting spore respiration, since we have found that the oxygen uptake of germinated spores is also considerably less sensitive to cyanide than that of vegetative cells.

The occurrence of combined flavin-adenine dinucleotide, together with the demonstration of a

diaphorase-like enzyme in spores of *B. subtilis* and *B. megatherium*, offers some support for the view that bacterial spores possess a respiratory mechanism involving a flavoprotein. We have found the oxygen uptake of resting spores in buffered glucose to be extremely low. If the uptake measured is, in fact, that of resting spores and not due to a small proportion of germinated forms, its cyanide-insensitive component may possibly be attributed to slow autoxidation of the diaphorase-like enzyme. If this enzyme is indeed diaphorase, it must be coupled with a readily autoxidized catalyst in germinated spores to account for their relatively high respiration rate. This catalyst may well be a haematin compound of comparatively low cyanide sensitivity.

In accounting for the low sensitivity of their respiration to cyanide, the possibility must not be ignored that spores may contain a large excess of heat-stable cytochrome oxidase (Militzer, Sondegger & Tuttle, 1950) over cytochrome *c* or some other intermediate constituent of the system. The amount of cytochrome *c* or other intermediate compound would then be the limiting factor in the oxidation, even when a large part of the cytochrome oxidase was inactivated by an inhibitor. Preliminary experiments to investigate this point have shown that both intact and broken resting spores of the three organisms used in this report will catalyse the oxidation of dimethyl *p*-phenylenediamine and quinol (Powell, 1952). These oxidations do not, however, show the characteristics of a typical cytochrome oxidase catalysis. Moreover, the oxidizing activity of spores disappears during 3–4 months' storage in water at room temperature, although viability and tendency to germinate in the chemically defined media are maintained. It seems possible, therefore, that an atypical cytochrome-cytochrome oxidase system may, at first, exist in resting spores, but that this system is not necessary to maintain viability or to promote the initial chemical changes involved in germination.

SUMMARY

1. Flavin-adenine dinucleotide has been shown to be present in all the cells tested, i.e. resting spores, germinated spores, and vegetative cells of laboratory strains of *Bacillus subtilis* and *B. megatherium*, and resting spores of *B. subtilis* N.C.T.C. 85.

2. During germination of spores of the laboratory strains of *B. subtilis* and *B. megatherium* in chemically defined media, no change in total flavin-adenine dinucleotide content, or in the relative amounts of free and bound dinucleotide occurred.

3. No L-amino-acid oxidase, D-amino-acid oxidase or xanthine oxidase activity could be demonstrated in resting spores of *B. subtilis* and *B. megatherium* or in germinated spores of *B. megatherium* and the laboratory strain of *B. subtilis*.

4. An enzyme capable of oxidizing reduced coenzyme I and reducing methylene blue occurred in all types of cell of *B. subtilis* and *B. megatherium*.

5. Spores of *B. megatherium* contained a heat-stable substance which inhibited reduction of methylene blue by heart diaphorase, the inhibition being prevented by the addition of large amounts (1 mg.) of coenzyme I.

6. No oxygen uptake could be measured with resting spores of the laboratory *B. subtilis* in phosphate buffer. The Q_{O_2} of these spores in buffered glucose, after preliminary heating at 60° for 15 min., was 0.42.

7. Oxygen uptake of *B. subtilis* and *B. megatherium* spores increased approximately 200 times following germination. This oxygen uptake was considerably less sensitive to cyanide than that of fully developed vegetative cells.

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Pseudo-Cholinesterase Activity in the Central Nervous System

By MARGERY G. ORD* AND R. H. S. THOMPSON

Department of Chemical Pathology, Guy's Hospital Medical School, London

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In an earlier report on the types of cholinesterases present in the tissues of the rat (Ord & Thompson, 1950), it was shown that whereas whole brain, striated muscle and suprarenal gland contain only small amounts of a 'non-specific' (Nachmansohn & Rothenberg, 1945) or 'pseudo'-cholinesterase (Mendel & Rudney, 1943a), the other tissues which were studied each showed a considerable level of activity of this type of esterase; stomach, liver, lung

and salivary gland were found to hydrolyse benzoylcholine and acetyl- β -methylcholine at approximately equal rates, while heart, intestinal muscle and mucosa, Harderian gland and skin contain predominantly a pseudo-cholinesterase, benzoylcholine being hydrolysed from two to six times more rapidly than acetyl- β -methylcholine.

Although no evidence could at that time be brought forward as to the physiological significance of this widely distributed pseudo-cholinesterase, it was decided to extend this study to human tissues,

* Present address: Department of Biochemistry, University of Oxford.

and because of the interest attaching to the high level of overall cholinesterase activity in the central nervous system, a start was made with human brain.

Although little work has been done on human brain in this respect, the brains of a number of other mammalian species have received attention. Nachmansohn (1939) demonstrated the varying ability of different areas of dog, ox, rabbit and human brain to hydrolyse acetylcholine and showed that the white matter of the central nervous system was less active than the grey. Birkhäuser (1940) also reported on the acetylcholine-hydrolysing activity of human brain. Turning to the different types of cholinesterase, Mendel & Rudney (1943b), using 'specific' substrates for 'true' and pseudo-cholinesterases (Mendel, Mundell & Rudney, 1943), stated that the brains of a number of vertebrate species contained only the true cholinesterase and no pseudo-cholinesterase (see also Nachmansohn & Rothenberg, 1944). Little (1948), working with preparations derived from whole mouse brain, also failed to demonstrate any significant amount of pseudo-cholinesterase. Contrary to the earlier views on the specificity of brain cholinesterase, Augustinsson (1948) reported that extracts of dog and elephant brain and also of the caudate nucleus of the bear split benzoylcholine, although at a very low rate. Zeller (1949) also found both types of esterase present in elephant brain, and in homogenates of whole rat brain Ord & Thompson (1950) demonstrated a measurable rate of hydrolysis of benzoylcholine, although amounting to only about 6% of the rate of hydrolysis of acetylcholine. In a detailed study of the dog's brain Burgen & Chipman (1951) have recently shown that benzoylcholine is hydrolysed at a relatively rapid rate by certain areas. Both types of cholinesterase had earlier been found to be present in peripheral nerve (Boell, 1945; Sawyer, 1946).

In view of our earlier findings, and since the different types of cholinesterase do not appear to have been studied in human brain, we decided to investigate the substrate specificity of the cholinesterases in this human tissue, and to attempt to characterize the enzymes further by summation studies and by a study of the sensitivity of the hydrolysis of different esters to inhibition by eserine, diisopropyl fluorophosphonate and the selective inhibitor Nu 1250 (see below) introduced by Hawkins & Mendel (1949). We have also extended some of these findings to rat, rabbit, guinea pig, cat and dog brains.

EXPERIMENTAL

Estimation of esterase activity. This was carried out manometrically at 38° and at pH 7.4, using the Warburg apparatus. All measurements were made in duplicate, and

were corrected for non-enzymic hydrolysis of the substrate. Esterase activity is expressed as $\mu\text{l. CO}_2/\text{g. wet wt. of tissue/hr.}$

Preparation of tissues. Human tissues were removed from cadavers as soon as possible after death; it was later found that storage of human nervous tissue at 0–4° for 3–5 days did not cause any significant loss of esterase activity.

The parts of the brain to be studied were dissected out, mopped free from adherent blood with filter paper, washed with 0.9% (w/v) saline, weighed and homogenized in 0.025M-NaHCO₃. The homogenates, whose concentrations were chosen to give suitable CO₂ evolutions when measured over 0–30 min., were added to the main bulbs of the Warburg flasks.

Brains from other species were obtained after the animals had been killed by CHCl₃, decapitation or air-embolism; the areas under study were then prepared and homogenized as above.

Substrates. Acetylcholine chloride (ACh), British Drug Houses Ltd.; acetyl- β -methylcholine chloride ([Me₃N⁺CH₂.CHMe.OAc] Cl⁻) (MCh), Savory and Moore Ltd.; benzoylcholine chloride (BCh), prepared by Dr A. H. Ford Moore, Experimental Station, Porton; propionylcholine perchlorate (PrCh), provided by Dr V. P. Whittaker, Department of Biochemistry, Oxford, and later synthesized by standard methods; butyrylcholine chloride (BuCh), British Drug Houses Ltd.; tributyrin (TB), British Drug Houses Ltd.

The choline esters were dissolved in 0.025M-NaHCO₃, immediately before use, the final concentrations used throughout being 0.015M for ACh and BCh, and 0.03M for MCh, PrCh and BuCh. The tributyrin was pipetted directly into the side arm of the Warburg flask (0.03 ml./flask), and was covered with a small volume of 0.025M-NaHCO₃.

Inhibitors. Eserine sulphate, British Drug Houses Ltd.; diisopropyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton; *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250), Hoffman la Roche Ltd.

The inhibitors were dissolved in 0.025M-NaHCO₃, immediately before use.

RESULTS

The relative activities of homogenates of different regions of human brain towards ACh, BCh, BuCh and MCh are shown in Table 1. The results given are the mean of at least two experiments with each tissue.

In agreement with the work of Nachmansohn (1939) and others, the rate of hydrolysis of acetylcholine varies widely according to the region studied, being high in the basal ganglia and other areas rich in synapses, but low in those parts consisting mostly of tracts of fibres.

When the activity towards acetyl- β -methylcholine is considered, it will be seen that those regions which are most active towards acetylcholine and which are rich in synapses (basal ganglia and grey matter) have also a high activity towards MCh, confirming the previous classification of brain cholinesterase as being mainly of the specific or 'true' type.

In all the regions studied, however, a measurable rate of hydrolysis of BCh was present. Here also variations in rate were found between preparations from different areas though the differences were smaller in degree than with either ACh or MCh. In regions rich in synapses, the activity towards BCh tended to follow the activity towards MCh, although it was in every case very much lower than the MCh

the rates of hydrolysis of non-choline esters by different regions of the brain with their cholinesterase contents (Table 2). The activity towards tributyrin was fairly constant in the areas of high synaptic density which were examined, but was much lower in the subcortical white matter. From a comparison with the results given in Table 1 the levels of TB hydrolysis do not appear to be related

Table 1. *Relative cholinesterase activities of different regions of human brain, using ACh, BCh, BuCh and MCh as substrates*

Region	Absolute activity (μ l. CO ₂ /g. (wet wt.)/hr.)				Activity as percentage of ACh activity			Ratio: MCh/BCh
	ACh	BCh	BuCh	MCh	BCh	BuCh	MCh	
Lentiform nucleus	24650	438	1392	17775	2	6	72	36
Caudate nucleus	16900	240	881	10790	1	5	64	64
Substantia nigra	5540	173	—	4110	3	—	74	25
Cerebellum	4540	152	1600	2887	3	35	64	21
Thalamus	1620	94	650	942	6	40	58	10
Red nucleus	1537	128	—	1354	8	—	88	11
Cortical grey matter								
Pre- and post-central gyri	476	35	204	260	7	43	55	8
Frontal cortex	439	31	—	230	7	—	52	7
Occipital cortex	322	31	—	170	10	—	53	5
Subcortical white matter								
Pre- and post-central gyri	275	91	594	82	33	216	30	0.9
Frontal lobe	523	131	1024	64	25	196	12	0.5
Occipital lobe	319	66	—	53	21	—	17	0.8

level. On the other hand, in the different areas of subcortical white matter which were examined, the rate of hydrolysis of BCh was in every experiment greater than that of MCh, and amounted to 21–33 % of the activity towards ACh. This finding was emphasized when BuCh was used as a substrate instead of BCh. Although this compound is very readily hydrolysed by pseudo-cholinesterases it is

either to the activity towards MCh or towards BCh or BuCh. The independence of the enzymes hydrolysing these choline and non-choline esters is confirmed both by summation experiments (Table 3) and by the use of eserine as a differential inhibitor, 10⁻⁶M-eserine sulphate producing 80–97 % inhibition of the hydrolysis of the choline esters, and only 2–22 % inhibition of the hydrolysis of TB (Table 4).

These results suggest that the white matter of the human cerebrum contains very much more pseudo-cholinesterase than the grey matter, although its tributyrinase content is lower. It was decided therefore to study the brains of other mammalian species to determine whether this predominant distribution of pseudo-cholinesterase in the white matter applies also to other species. Results obtained with preparations from the grey matter of the cerebral cortex and the subcortical white matter from five species are shown in Table 5; results obtained with human brain are included for comparison.

It will be seen that in all the species studied the rate of hydrolysis of BuCh by subcortical white matter was greater than that of MCh, and in every species except the dog BuCh was hydrolysed more rapidly by subcortical white matter than by the grey matter of the cerebral cortex.

Table 2. *Hydrolysis of tributyrin by different regions of human brain*

Region	Activity (μ l. CO ₂ /g. (wet wt.)/hr.)
Lentiform nucleus	3972
Caudate nucleus	3393
Cerebellum	2132
Thalamus	2353
Cortical grey matter (pre- and post-central gyri)	2640
Subcortical white matter (pre- and post-central gyri)	802

only attacked slowly by the true cholinesterase. It will be seen from Table 1 that human subcortical white matter hydrolyses BuCh at more than twice the rate of ACh hydrolysis and more than seven times the rate for MCh.

A number of experiments have also been carried out with tributyrin as substrate in order to compare

Table 3. *Summation experiments with choline esters and tributyrin as substrates for human-brain preparations*

Enzyme source	Hydrolysis rate ($\mu\text{l. CO}_2/\text{g./hr.}$)				'Summation' (%)
	Choline ester	Tributyrin	Both esters		
			Found	Calc.	
	ACh				
Subcortical white matter	780	928	1496	1708	88
Cortical grey matter	585	3376	3580	3961	90
	MCh				
Cerebellum	3340	2660	5960	6000	99
	BuCh				
Subcortical white matter	817	1158	1813	1975	92
Lentiform nucleus	1202	4444	6180	5646	110
Caudate nucleus	938	3396	4620	4334	107

Table 4. *Effect of eserine (10^{-6}M) on the hydrolysis of choline esters and tributyrin by human-brain homogenates*

Enzyme source	Inhibition (%)	
	Choline ester	Tributyrin
	BuCh	
Lentiform nucleus	82	8
Caudate nucleus	96	2
Subcortical white matter	80	8
	ACh	
Cerebellum	93	11
Cortical grey matter	97	22

Table 5. *Distribution of cholinesterases in the grey and white matter of the cerebrum of different mammalian species*

Enzyme source	Absolute activity ($\mu\text{l. CO}_2/\text{g./hr.}$)			Activity as percentage of ACh activity		Ratio: MCh/BuCh
	ACh	BuCh	MCh	BuCh	MCh	
		Cortical grey matter				
Human	476	204	260	43	55	1.3
Rabbit	8340	1981	6710	24	81	3.4
Rat	6100	1935	4555	32	75	2.4
Guinea pig	4370	1009	2720	23	62	2.7
Cat	2340	117	2295	5	98	20
Dog	1799	1173	1063	65	59	0.9
		Subcortical white matter				
Human	275	594	82	216	30	0.1
Rabbit	7790	9084	2387	117	31	0.3
Rat	6525	3257	2389	50	37	0.7
Guinea pig	7145	18925	1564	266	22	0.1
Cat	2350	4550	549	194	23	0.1
Dog	328	595	151	181	46	0.3

Characterization of the pseudo-cholinesterase in white matter

Although BCh and BuCh have been shown to be 'specific' substrates for pseudo-cholinesterases from other sources it was thought advisable to characterize more exactly the enzyme in white matter hydrolysing these substrates.

Summation experiments with BCh and MCh confirmed that these substrates were hydrolysed by different enzymes; for example, the rates of hydrolysis found in one experiment with human white matter were 64, 97 and 139 $\mu\text{l. CO}_2/\text{g./hr.}$ for BCh, MCh and BCh + MCh respectively, i.e. 87% 'summation' was obtained.

As the white matter in all the species studied

always showed considerable true cholinesterase activity, a preparation from human subcortical white matter was partially purified by centrifugation of a water homogenate, the greater part of the true cholinesterase being discarded in the centrifuged supernatant (Ord & Thompson, 1951). Table 6 gives a comparison of the relative rates of

Table 6. *Comparison of the relative rates of hydrolysis of different esters by human white matter (cerebrum) and by purified human plasma pseudo-cholinesterase*

Substrate	Human white matter		Human plasma*
	Unpurified	Partially purified	
Acetylcholine	100	100	100
Benzoylcholine	33	31	36
Propionylcholine	157	160	—
Butyrylcholine	216	202	208
Acetyl- β -methylcholine	30	12	1
Tributyrin	293	—	45

* Data of Adams & Whittaker (1949).

hydrolysis of a number of different esters by the unpurified and partially purified preparations from white matter with those found by Adams & Whittaker (1949) for the purified cholinesterase of human plasma.

The relative rates of hydrolysis of ACh, BCh and BuCh agree well with those found for the purified plasma enzyme, although the unpurified brain preparation naturally hydrolyses MCh and TB very much more rapidly. The similarity between the BCh-hydrolysing enzyme in white matter and the plasma pseudo-cholinesterase is further borne out when the concentrations of DFP required to inhibit the enzyme in these two sources are compared (Table 7). This table also shows that the sensitivity

Table 7. *Concentrations (I_{50}) of DFP required to produce 50% inhibition of the cholinesterases of human white matter, plasma and erythrocytes*

Enzyme source	Substrate	I_{50} ($\times 10^{-9}$ M)
Human white matter	BCh	2.0
Human white matter	BuCh	1.4
Human plasma*	BCh	2.6
Human white matter	MCh	125
Human erythrocytes*	MCh	130

* Data of Adams & Thompson (1948).

of the MCh-hydrolysing enzyme to inhibition by DFP closely resembles that of the true cholinesterase in human erythrocytes, and provides further evidence for the presence of these two types of cholinesterase in white matter.

The finding that, of the five choline esters studied (Table 6), butyrylcholine is hydrolysed most rapidly

suggests that this enzyme in human subcortical white matter may be of the butyryl-cholinesterase type (Sturge & Whittaker, 1950), very similar in those of its properties which have been examined to the pseudo-cholinesterase in plasma. In the white matter of rat brain the MCh/BuCh ratio was significantly higher than that obtained for the other species studied (Table 5). Since we had earlier (Ord & Thompson, 1951) found that the purified pseudo-cholinesterase in rat heart is a 'propiono-cholinesterase', hydrolysing PrCh almost twice as rapidly as BuCh, we tested a rat-brain preparation (white matter) with PrCh; we found that the PrCh/BuCh ratio was 3.2, indicating that the predominant cholinesterase in this rat tissue may also be of the propiono-cholinesterase type rather than the butyryl-cholinesterase type present in the other species.

Quantitative aspects of the distribution of cholinesterases in human brain

Since the different regions of the brain which we have studied all show activity towards both BCh and MCh, any quantitative consideration of the relative amounts of pseudo-cholinesterase in the different areas is complicated by the fact that the level of the true cholinesterase varies so very widely in different regions. It is for this reason that detailed consideration has so far been restricted to the grey and white matter of the cerebrum, in each of which areas the overall activity towards ACh is of the same order. It will have been noticed, however (Table 1), that the levels of hydrolysis of BCh and BuCh are very high indeed in the basal ganglia, cerebellum and thalamus. Moreover, in these regions the activity towards these substrates tends to parallel that towards MCh. Preparations from the caudate nucleus of the rabbit and the rat were also studied, and in these species also a high level of true cholinesterase was found to be associated with a high rate of BuCh hydrolysis.

These facts suggested that the very large amount of true cholinesterase in these areas might be contributing to the hydrolysis of BCh and BuCh. Adams (1949) has reported that the purified true cholinesterase from human erythrocytes hydrolyses BuCh and BCh at 5 and 1.5% respectively of the rate at which it hydrolyses MCh. If this also applies to the true cholinesterase in brain it would indicate that a considerable contribution to the high rate of hydrolysis of BuCh by these areas would be due to the splitting of this substrate by the very high concentration of true cholinesterase present.

Experiments were therefore carried out with the selective inhibitors DFP and Nu 1250 to compare the sensitivity of BuCh hydrolysis to these compounds in a region poor in true cholinesterase (subcortical white matter) with regions very rich in this enzyme

(lentiform and caudate nuclei and cerebellum). The results of these experiments are given in Table 8.

It will be seen that the hydrolysis of BuCh by subcortical white matter is inhibited 96 and 8% respectively by these concentrations of DFP and Nu 1250, indicating that in this region the hydrolysis is almost entirely brought about by an esterase sensitive to DFP but insensitive to Nu 1250, i.e. a pseudo-cholinesterase. In the areas very rich in true cholinesterase, however, BuCh hydrolysis is only partially inhibited (36–51%) by either of these

Table 8. *Inhibition of human-brain cholinesterases by DFP and Nu 1250*

Tissue	Substrate	Inhibition (%) produced by	
		$0.9 \times 10^{-8} \text{ M}$ DFP	$1.67 \times 10^{-6} \text{ M}$ Nu 1250
Subcortical white matter	MCh	3	100
	BuCh	96	8
Lentiform nucleus	MCh	18	97
	BuCh	36	50
Caudate nucleus	MCh	10	98
	BuCh	51	45
Cerebellum	MCh	9	—
	BuCh	49	—

two compounds. Since the concentrations of the two inhibitors which we have used are sufficient to cause almost complete inhibition of the pseudo- and true cholinesterases respectively, these results suggest that in these regions BuCh hydrolysis is being brought about to a significant degree by both enzymes, so that a measure of its rate of hydrolysis by these parts does not give a valid assessment of the amount of pseudo-cholinesterase present.

DISCUSSION

The results described above indicate that all the areas of human brain which have been studied contain, in addition to their true cholinesterase, an enzyme that hydrolyses both BCh and BuCh but not MCh. From its substrate specificity and from studies of sensitivity to inhibition by eserine and DFP it is clear that this enzyme is distinct from the tributyrinase which is present in these preparations, and is similar to, if not identical with, the pseudo-cholinesterase present in human plasma.

The distribution of this enzyme in the different areas of the brain raises some interesting points. The absolute level of BuCh hydrolysis is high in the lentiform and caudate nucleus, the cerebellum and the thalamus; but, as pointed out above, it would seem that a considerable part of this activity is due to hydrolysis of BuCh by the relatively enormous concentration of true cholinesterase present in these parts. However, a comparison of the grey and white

matter of the human cerebrum, areas having ACh-hydrolysing activities of the same order, shows that the subcortical white matter has very much greater pseudo-cholinesterase activity than the grey; the extremely low level of true cholinesterase activity in this region indicates that it is not playing any significant part in the BuCh hydrolysis. In each of the other species studied, the level of pseudo-cholinesterase is also considerably greater than that of the true cholinesterase in the white matter, and in every species except the dog it is also present in much larger amounts in the subcortical white matter than in the cortical grey. The high level of this enzyme in the white matter of the cerebrum suggests that it may be associated particularly with the myelinated fibres of the nervous system, and experiments are now in progress with spinal cord and with myelinated peripheral nerves to determine whether this association holds also for the myelin-containing regions of these tissues. It is of interest that Burgen & Chipman (1951), in their recent study of dog brain, have concluded that in this species also the fibre tracts in general have a high BCh-hydrolysing activity.

These findings naturally raise the question of the function of this pseudo-cholinesterase, particularly in the white, myelinated areas of the central nervous system. One possibility is that it may be connected with processes occurring in the myelin sheaths or the neuroglial elements of the nervous system rather than directly with the conducting properties of the axis cylinders. Support is given to this suggestion by the findings of Sawyer (1946), who reported that Wallerian degeneration of guinea pig peripheral nerves, with complete disintegration of the axis cylinders, is not associated with any loss of pseudo-cholinesterase activity in the affected nerves, even though the true cholinesterase falls by about 60%. In this connexion it may be of interest to record some preliminary observations which we have made with tri-*o*-cresyl phosphate (Earl & Thompson, 1952). This substance was shown by Smith & Lillie (1931) to produce areas of demyelination both in the spinal cord and in peripheral nerves. Bloch in 1941 had demonstrated *in vitro* its anti-cholinesterase properties. Despite this latter finding, injection of the compound into animals does not, in most of the species studied, give rise to any signs of acetylcholine accumulation. Tri-*o*-cresyl phosphate is, however, only very slightly soluble in water, although it is freely lipid-soluble, and together with Dr C. J. Earl we have shown that it is a highly selective inhibitor for the pseudo-cholinesterase in human brain and plasma; *in vitro* we have found that concentrations of $1.4 \times 10^{-4} \text{ M}$ caused 60–90% inhibition of the pseudo-cholinesterase in human cerebrum, but only 0–7% inhibition of the true cholinesterase in this site or in human erythrocytes. It

must be stressed that these latter findings are of a preliminary nature and are as yet unsupported by any *in vivo* work, so that although we have evidence of an association of pseudo-cholinesterase activity with the white fibre tracts of the central nervous system we are at present unable to reach any conclusions as to its physiological function in these areas.

SUMMARY

1. The relative rates of hydrolysis of a number of different choline esters by preparations from different areas of human and other mammalian brains have been determined.

2. In all the areas studied, a measurable degree of

hydrolysis of benzoylcholine and butyrylcholine was observed.

3. The benzoylcholine-hydrolysing enzyme present in human nervous tissue appears to be a 'butyro-cholinesterase' closely resembling the pseudo-cholinesterase present in human plasma.

4. The white fibre tracts of the human cerebrum were found to contain significantly more of this pseudo-cholinesterase than the grey matter.

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The Metabolism of Short-Chain Fatty Acids in the Sheep

1. FATTY ACID UTILIZATION AND KETONE BODY PRODUCTION BY RUMEN EPITHELIUM AND OTHER TISSUES

By R. J. PENNINGTON

Rowett Research Institute, Bucksburn, Aberdeenshire

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Large quantities of volatile fatty acids, chiefly acetic, propionic and butyric acids, are produced by the bacterial decomposition of the foodstuffs in the rumen of the sheep and other ruminants and are absorbed directly from this organ (see review by Elsdon & Phillipson, 1948). The problem of assessing the relative amount of each acid produced is not altogether straightforward, owing to differing rates of absorption. Application of chromatography has led to the identification of smaller quantities of

higher isomers and of formic acid (Gray, Pilgrim, Rodda & Weller, 1951). Subsidiary quantities of volatile fatty acids are also produced in and absorbed from the large intestine.

Although the production of volatile fatty acids in the gut is not confined to ruminants (Phillipson, 1947*a*), the latter present a special problem since it is highly probable that these compounds, previously considered as by-products of digestion, form their chief source of energy. The amount of sugar available

the other columns have been corrected by the addition of the figures in column 4. In this and in subsequent tables the amounts of fatty acid utilized are calculated to 100 mg. of tissue (dry weight) in order to eliminate the effect of any variations in the

Table 4. *Influence of pH of medium upon uptake of volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39.5° in Ringer-phosphate containing 100 μ moles of fatty acid as Na salt.)

pH	Fatty acid lost (μ moles)			Volatile acid production in control (μ moles)
	Acetic	Propionic	Butyric	
7.2	4.9	8.6	30.1	1.1
6.5	5.6	2.1	16.1	1.0
5.8	3.9	0.5	10.3	0.5

wet weight/dry weight ratio. Such variations never exceeded 10% of the ratio in any one experiment. The loss of dry weight during incubation of the tissue amounted to less than 10%, so that the figures are slightly higher than if based on initial dry weight.

It is clear that in every experiment the disappearance of butyrate exceeded that of the other two.

The activity of the tissue appeared to be well maintained during the 3 hr. incubation, since when incubated for only 1 or 2 hr. the amount of acid disappearing was correspondingly less. A longer incubation period was not used owing to the risk of bacterial growth in the flasks.

Some experiments were carried out in which the conditions of incubation were altered. When the concentration of fatty acids was increased eightfold the amounts disappearing were increased 3–5 times. Table 4 shows the effect on the disappearance rates of lowering the pH of the medium by the addition of hydrochloric acid. (The additional chloride thus introduced was very small compared to that already present in the medium and would not, in itself, be expected to influence the results.)

The effect of using bicarbonate buffer of approximately the same pH (Krebs-Ringer-bicarbonate, Umbreit *et al.* 1945) instead of phosphate buffer, in an otherwise identical medium, was also determined. Ciaranfi (1936) found a more rapid utilization of butyric acid by liver slices in a phosphate-buffered than in a bicarbonate-buffered medium. The results of three experiments with different sheep are shown in Table 5.

Table 5. *Influence of buffer and of carbon dioxide upon uptake of volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39.5° in Ringer-phosphate or Ringer-bicarbonate containing 100 μ moles of fatty acid as Na salt.)

Sheep no.	Buffer	Gas phase	Fatty acid lost (μ moles)		
			Acetic	Propionic	Butyric
1	Phosphate	O ₂	5.6	7.8	25.3
	Bicarbonate	O ₂ + 5% CO ₂	5.0	13.7	24.6
2	Phosphate	O ₂	5.6	10.4	28.9
	Bicarbonate	O ₂ + 5% CO ₂	3.6	20.3	29.9
	Phosphate	O ₂ + 5% CO ₂	6.5	18.8	31.4
3	Phosphate	O ₂	4.4	1.2	27.3
	Phosphate	O ₂ + 5% CO ₂	6.0	6.0	27.1

Although the mean of the propionate figures is greater than that of the acetate, this is of little importance owing to the very great variation among the propionate results. (It is of interest that the variance of the latter is 26.98, compared to 2.24 for acetate; a variance ratio of 12 indicates a highly significant difference ($P=0.002$) between the two variances.)

In one of the experiments the rates of distillation of the unused acids were recorded by collecting the distillates in 10 ml. fractions and titrating each fraction. In each case the observed rate corresponded exactly with that of an equivalent quantity of the original fatty acid, thus providing a check that the estimated volatile acid in the experiments represented entirely the unused portion of the original 100 μ moles and not a different volatile acid produced from it by the tissue.

In the first experiment the substitution of bicarbonate for phosphate had a striking effect upon the utilization of propionate, the disappearance rate of which was nearly doubled. This effect was confirmed in the second experiment, where, in addition, the disappearance rates were determined using phosphate buffer but gassing the flasks with oxygen + 5% of carbon dioxide, as used with the bicarbonate buffer. The elevation in the rate of disappearance of propionate was maintained. In the case of the other two fatty acids there were no such marked differences resulting from the presence of carbon dioxide. The relatively small effect upon acetate utilization resulting from the presence of carbon dioxide may not be significant. This will be investigated further.

The important question of the fate of the fatty acids metabolized by the rumen epithelium was

partly answered by testing the media, after incubation, for ketone bodies. Application of Rothera's test invariably gave a strong response following the disappearance of butyrate. A positive response was obtained also on the acetate media but none in the case of propionate, although the controls incubated without fatty acid always gave a little colour. In two subsequent experiments determinations of ketone bodies were carried out (Table 6: results with two sheep).

Table 6. *Production of ketone bodies from volatile fatty acids by rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. at 39.5° in Ringer-phosphate containing 100 μ moles of fatty acid as Na salt. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.)

Ketone bodies produced (μ moles) in presence of			
Acetic	Propionic	Butyric	Control
1.8 (76)	-2.7	22.7 (77)	4.4
0.4 (19)	-2.7	18.9 (65)	3.3

The figures (calculated as usual to 100 mg. dry weight) are corrected for the ketone production in the controls. The amount of fatty acid utilized was also determined (nos. 9 and 10 in Table 3) and the proportion of this appearing as ketone bodies calculated (assuming that one molecule of ketone is derived from two molecules of acetic acid or one molecule of

butyric acid). It can be seen that more than one-half of the butyric acid disappearing could be accounted for as ketone bodies. Of the total ketone body production less than one-quarter was present as β -hydroxybutyric acid. In the case of acetate the relatively small difference from the controls makes the validity of the calculated percentages doubtful, since it is impossible to say whether the endogenous reactions producing ketone bodies in the controls would continue at the same rate in the presence of added substrate. If not, the corrections are not justified and the figures should consequently be higher. The appearance of ketone bodies in the propionate medium was in each case less than that in the control medium.

In another experiment the initial concentration of butyrate was halved (50 μ moles/flask). The proportion of that lost appearing as ketone bodies was 65 % as compared to 70 % in a parallel run employing the usual concentration of butyrate. In the experiment employing eightfold concentrations of the fatty acids, mentioned above, 59 % of the metabolized butyric acid was converted to ketone bodies:

In further experiments the ability of other tissues of the sheep to utilize the same fatty acids under identical conditions was determined; the ketone body production was measured in each case. The results are summarized in Table 7. Both fatty acid disappearance and ketone production are corrected by the control figures, as in previous tables.

Table 7. *Volatile fatty acid uptake and ketone body production by various sheep tissues*

(Tissue (2 g.) incubated for 3 hr. at 39.5° in Ringer-phosphate containing 100 μ moles of fatty acid as Na salt. Results given as μ moles. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.)

Tissue	Acetic		Propionic		Butyric		Control	
	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Volatile acid produced	Ketone bodies produced
Reticulum epithelium	5.3	1.5 (56)	3.9	-0.3	23.8	17.5 (73)	1.3	0.6
Omasum epithelium	2.5	0.2 (16)	1.5	-0.2	10.8	5.3 (49)	0.3	0.5
Abomasum epithelium	4.6	1.9 (83)	1.4	-0.8	30.0	24.2 (81)	0.0	1.7
Caecum epithelium	1.6	0.3 (38)	0.7	-0.2	8.6	4.0 (47)	0.8	1.0
Liver	18.8	-0.4	14.2	-0.4	18.2	3.3 (18)	0.2	0.9
Kidney cortex	20.2	2.2 (22)	13.7	0.5 (8)	16.9	5.3 (31)	0.0	0.4
Heart muscle	12.6	0.4 (6)	10.0	0.2 (4)	9.4	1.1 (12)	1.0	0.3
Brain	10.8	-0.9	6.7	-0.9	10.0	1.0 (10)	1.2	1.8
	13.0	0.3 (5)	8.8	0.0	11.6	0.3 (3)	0.7	1.2
	6.9	0.9 (26)	5.9	0.6 (20)	20.0	14.8 (74)	0.6	0.0
	15.8	1.6 (20)	22.3	-3.8	23.9	16.6 (69)	0.9	1.9
	42.6	0.0	43.8	0.0	38.5	3.8 (10)	0.0	1.3
	54.5*	0.6 (2)	60.9	0.0	41.2	8.6 (21)	0.0	0.4
	0.0	—	0.0	—	1.4	0.1 (7)	0.4	0.2
	4.2	0.1 (2)	0.4	0.0	1.1	0.2 (18)	0.6	0.0
	0.0	—	0.0	—	0.0	—	1.2	—
	0.0	—	0.0	—	0.0	—	2.1	—

* Only 1 g. of tissue used.

The figures in each line in the table were obtained on tissue from a different sheep. The epithelial tissues were prepared in the same way as the rumen epithelium; although this was rather difficult in the case of epithelium from the abomasum and caecum owing to their greater fragility, satisfactory results were obtained after a little practice. The other tissues were sliced in the standard manner. As a

ketone bodies. On the other hand, kidney cortex, and epithelium from the abomasum (the true stomach of the sheep) and from the caecum, utilized acetate at least as rapidly as butyrate and, moreover, produced a relatively smaller amount of ketone bodies. The results with heart-muscle slices were low and less consistent. Slices of whole brain did not utilize any of the three fatty acids.

Table 8. *Volatile fatty acid uptake and ketone body production by rat tissues*

(Liver tissue (2 g.) or 1 g. of kidney tissue incubated for 3 hr. at 39.5° in Ringer-phosphate containing 100 μ moles of fatty acid as Na salt. Results given as μ moles. The figures in parentheses are the percentages of the maximum possible production of ketone bodies from the fatty acid utilized.)

	Acetic		Propionic		Butyric		Control	
	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Fatty acid used	Ketone bodies produced	Volatile acid produced	Ketone bodies produced
Liver	27.0	3.1 (23)	23.1	- 2.1	31.5	19.6 (62)	0.7	7.7
Kidney	92.5	1.2 (3)	56.8	0.9 (3)	58.0	6.0 (10)	0.9	0.0

Table 9. *Changes in blood volatile fatty acids and ketone bodies resulting from the administration of fatty acids into the rumen*

(Results expressed as μ moles/100 ml.)

Liquid in rumen	Rumen blood		Carotid blood	
	Ketone bodies	Fatty acid	Ketone bodies	Fatty acid
Exp. 1				
Water	48	77	58	27
Sodium butyrate, 0.15M	222	118	199	73
Water	83	46	86	41
Sodium propionate, 0.15M	83	369	86	100
Water	60	46	58	50
Sodium acetate, 0.15M	78	611	76	114
Water	19	68	48	45
Sodium butyrate, 0.15M	219	123	191	64
Exp. 2				
Water	47	50	47	50
Sodium butyrate, 0.15M	310	375	266	146
Water	110	32	95	27
Sodium propionate, 0.15M	43	565	43	100
Water	24	27	43	27
Sodium acetate, 0.15M	100	1088	47	142
Water	33	32	47	36
Sodium butyrate, 0.15M	195*	320*	134*	96*
	334	246	248	50

* Blood samples taken after 5 min. All other samples taken 30 min. after administration.

check, in each case, rumen epithelium from the same sheep was incubated with butyrate; in every case the amount of butyrate utilized fell within the range of values for butyrate seen in Table 3 and the proportion converted to ketone bodies ranged from 59 to 74%. Inspection of Table 7 shows that epithelium from the reticulum and omasum and also liver tissue behaved in a similar manner to rumen epithelium in that they utilized butyrate most readily and converted a large proportion of it into

For comparison exactly similar experiments were made with liver and kidney tissue from adult male rats. The results (Table 8), which concord with those reported by other workers, show no important differences from those obtained with the corresponding tissues of the sheep (Table 7).

The production of ketone bodies on such a scale by the epithelia of sheep fore-stomachs was rather unexpected, and it was considered desirable to try to confirm this by an *in vivo* experiment. For this

purpose a ewe with a permanent rumen fistula was anaesthetized by intravenous injection of Nembutal and operated upon in the manner described by Masson & Phillipson (1951) (the oesophagus and reticulo-omasal orifice were closed by ligatures and the carotid artery and posterior rumen vein exposed for withdrawal of blood samples). The rumen was emptied and washed clean with warm water. Blood samples were withdrawn for ketone body and volatile acid determination. Portions (3 l.) of each of the liquids indicated in Table 9, warmed to about 40°, were placed in the rumen in the order given in the table. Each was allowed to remain for half an hour, blood samples were then taken and the rumen washed out with warm water before the next insertion. Table 9 records the changes in ketone body and volatile acid levels of the blood in the rumen vein and carotid artery. The experiment was repeated on another sheep; in this case blood samples were taken at 5 min., as well as at 30 min., after the final addition of butyrate.

It may be seen that in each experiment the presence of butyrate in the rumen led to several-fold increases in the level of ketones in the blood. In every case the increase was greater in the blood draining the rumen than in the arterial blood, as would be expected if ketone bodies were produced in the rumen wall. A smaller rise of blood ketone bodies occurred with acetate in the rumen, whilst propionate produced no increase in the first experiment and a pronounced decrease in the second. In the first experiment each of the four solutions, after withdrawal from the rumen, was also analysed for ketone bodies. A total of 44.5 and 22.2 mg. respectively (as β -hydroxybutyric acid) was found in the butyrate solutions and 9.0 mg. in the propionate solution. No ketone bodies appeared in any of the other solutions.

DISCUSSION

The marked ability of the epithelia of sheep stomachs to metabolize the lower fatty acids, shown in these experiments, substantiates the conclusions drawn from previous work that an appreciable portion of the butyric acid absorbed is removed before it can enter the circulation. The results also show that this may apply, though to a lesser extent, to propionic and acetic acids. Although too much reliance cannot be placed on the quantitative significance of such *in vitro* studies, it is perhaps of interest to compute that the mean value for butyrate loss in Table 3 would represent a utilization rate of about 0.3–0.5 g. of butyric acid/hr. by the epithelium of the whole rumen, and correspondingly more than this when the reticulum and omasum are also taken into account. The concentration of butyrate used in these experiments is of the order of that

normally found in rumen liquor. These epithelia are not glandular and hence would not require the energy of oxidation of the fatty acids for secretory purposes. It is possible, however, that such energy may be utilized in connexion with 'active absorption' of substances from the rumen.

The fact that such a large proportion of the butyric acid was converted to ketone bodies by these tissues is also of considerable interest. Ketone body production on an important scale is generally acknowledged to be confined to the liver in the common laboratory animals. In the experiments reported in this paper, the epithelia of the fore-stomachs of the sheep were about as active, weight for weight, as liver tissue in the production of ketone bodies. The total wet weight of these epithelia was determined in one animal and found to be over half the weight of the liver, hence these tissues must be considered as important sources of ketone bodies in the sheep. It is possible that such a supplementary production of ketone bodies, which are known to be utilized by other tissues (Breusch, 1948), assists the animal in dealing with the large fatty acid intake from these organs. However, it remains to be seen whether such speculations are valid under normal conditions where mixtures of fatty acids are absorbed from the rumen.

On comparing the activities of all the tissues studied it is of interest to note that only the tissues which converted a large proportion of the butyrate into ketone bodies showed a marked preference for this fatty acid over the other two. The inability of the sheep-brain tissue to metabolize fatty acids is in accordance with the failure of other workers to observe the oxidation of acetate by brain tissue of other animals and of Quastel & Wheatley (1933) to observe the oxidation of butyrate. It appears that brain tissue may be unique in this respect.

The suggestion of McClymont (1951) that enzymes for metabolizing propionic acid in the sheep are concentrated in the liver is not borne out here. McClymont quotes, in support, the observation of Graffin (1948) that a cyclophorase preparation from rabbit kidney was incapable of oxidizing propionic acid although acetic acid was readily oxidized; both acids were readily oxidized by the liver preparation. In the present experiments propionate was removed most rapidly by kidney and was utilized by all the tissues with the exception of brain and possibly of cardiac muscle. The fate of the propionate is an important question which is being further studied. As mentioned previously, it seems highly probable, from present knowledge of ruminant digestion, that propionate may be the main source of carbohydrate for these animals. It has been demonstrated (Hitchcock & Phillipson, 1947b) that the administration of propionate to the phlorrhizinized sheep leads to the excretion of an

equivalent amount of glucose in the urine. It will be of interest to see whether the direct conversion of propionate to carbohydrate by sheep tissues can be demonstrated *in vitro* and, if so, whether this process is confined to the liver. The observation that the presence of carbon dioxide enhances the utilization of propionate points to a possible fixation of carbon dioxide in the metabolism of the latter. Carboxylation to succinate followed by carbohydrate synthesis would seem an obvious possibility, although there is no good evidence that propionate can be converted directly to succinate in living tissues.

An important difference between the experimental conditions here used and the conditions *in situ* is that in the latter case the tissues usually have available a mixture of the fatty acids. One possibility then is that propionate could be oxidized simultaneously with the other fatty acids and help to promote complete oxidation of the latter instead of ketone body formation. Quastel & Wheatley (1933) found that propionate inhibited ketone body formation from butyrate by liver slices, although their results could be explained simply by selective utilization of propionate. Jarrett & Potter (1950) in the paper already mentioned suggest that propionate, by conversion to pyruvate, may facilitate the metabolism of acetate. The modern concept of antiketogenesis, namely, the provision of pyruvate, which, by carboxylation to oxaloacetate increases the operation of the Krebs cycle can only be applied to tissues with β -carboxylase activity (Baldwin,

1949). It will be of interest to see whether antiketogenesis can be demonstrated in the rumen epithelium.

SUMMARY

1. The ability of rumen epithelial tissue of the sheep to metabolize butyric acid and, to a lesser extent, acetic and propionic acids, under aerobic conditions has been demonstrated; these compounds are amongst the chief products of fermentation in the rumen.

2. A large proportion of the butyric acid was converted to ketone bodies, mainly acetoacetic acid, by this tissue. Ketone bodies were formed also from acetic acid, but not from propionic acid.

3. The utilization of propionic acid was more rapid under an atmosphere containing 5% of carbon dioxide than under an atmosphere of oxygen.

4. The utilization of the volatile fatty acids by other sheep tissues has also been studied. Ketone bodies were produced from butyric acid by the epithelium of the reticulum and of the omasum as well as by liver tissue; all these tissues metabolized butyric acid more rapidly than acetic or propionic acids. The epithelia of the abomasum and caecum, and kidney tissue, did not show this preference for butyrate and also produced relatively less ketone bodies.

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Chemical Composition of a Sample of Mycelium of *Penicillium griseofulvum* Dierckx

By W. R. SMITHIES

Rothamsted Experimental Station, Harpenden, Herts

(Received 18 October 1951)

Both fungal and bacterial processes occur in the formation of soil organic matter (Waksman, 1938), which is itself composed, at least in part, of living micro-organisms and those parts of their remains which are resistant to chemical and enzymic decomposition. A knowledge of the composition of micro-organisms should, therefore, help to shed some light on the constitution of soil organic matter. Whilst much work has been done on the isolation and characterization of low molecular weight constituents of fungi, there are no analyses of mycelium in which the various macromolecular compounds are well defined.

A variety of carbohydrate preparations have been isolated from fungal tissue (Norman, Peterson & Houtz, 1932), and it has long been recognized that the cell wall of many fungal species is chitinous (Norman & Peterson, 1932; Brian, 1949). Protein concentrates have been extracted by Gorgica, Peterson & Steenback (1934) and Bohonos, Woolley & Peterson (1942). Takata (1929), and Akasi (1939) isolated nucleic acid preparations. Claims have been made for the isolation of 'crude lignin' or 'lignin-like complexes' by Thom & Phillips (1932), Phillips (1938) and Pinck & Allison (1944). Porges (1932) and Schulz (1937) studied the effect of cultural conditions on mycelial composition but, for the most part, their fractions are quite arbitrary and sometimes misleading. The literature is summarized and discussed by Foster (1949).

In this paper various analytical methods have been studied from the point of view of their usefulness in the determination of the chemical components, particularly the macromolecular ones, of a sample of mycelium of *Penicillium griseofulvum* Dierckx, grown as a surface pad. This is not a normal or important components of the soil flora, but was chosen for this work, which will be used as a model for future investigations on the predominant component of the soil, because it was readily available.

No precautions were taken to separate spores, or to avoid autolysis, and values given are by no means claimed to be typical of mould mycelium.

MATERIALS AND GENERAL METHODS

Culture of mycelium. I am indebted to Mr P. J. Curtis of the Butterwick Research Laboratories of Imperial Chemical

Industries for the following information. Culture: *P. griseofulvum* Dierckx (BRL 374) (ex Prof. H. Raistrick no. P 38). Medium: Czapek-Dox (Raistrick), containing 7.5% crude glucose and 0.1% peptone. The mould was grown in earthenware culture vessels of the type described by Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey (1941) using 1 l. medium in each and an incubation period of 14 days.

Preparation of mycelium. The mycelium, received a few hours after harvesting, was spread thinly on sheets of paper, and by the following day most of the moisture had evaporated, but although allowed to dry at room temperature for over a month, it never became sufficiently crisp to powder satisfactorily in a small C. and N. (Christy & Norris, Chelmsford) laboratory mill (0.5 mm. sieve). However, there was no difficulty with mycelium that was dried thus for only 3 or 4 days, if placed in the hot room at 40° for several hours before milling.

A portion of the powdered mycelium (*M*) was exhaustively extracted in a Soxhlet apparatus with light petroleum (b.p. 40–60°), ether, ethanol, acetone and chloroform, in that order, and again dried in air (*Ms*). Subsequently it was found that acetone, ethanol and chloroform alone were as effective and light petroleum or ether extracted no more material.

A portion of *Ms* was extracted twice with cold water (10 vol. for 30 min. with stirring) and then three times with the beaker standing in a boiling-water bath. The residual mycelium was exhaustively extracted with ethanol and air-dried (*Msw*).

Dry matter. Weighed samples were dried at 100–105° overnight and reweighed.

Total nitrogen. This was determined by a micro-Kjeldahl method using $\text{SeO}_2 \cdot \text{CuSO}_4 \cdot 5\text{H}_2\text{O} : \text{K}_2\text{SO}_4 : 1 :: 1 : 8$ as catalyst.

NH₃ nitrogen. This was estimated by microdiffusion in a Conway dish followed by nesslerization (Conway, 1947).

α -Amino-acid N was determined by the ninhydrin-titration method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2.5 and a reaction time of 8 min.

Total P. This was estimated by a modification of the method of Kuttner & Lichtenstein (1932).

Carbohydrate. This was determined by the colour given on heating with orcinol in H_2SO_4 using a glucose standard (Pirie, 1936). The intensity of colour was measured with a photoelectric colorimeter (the Evans Electro-selenium Portable model, using Ilford filter 622) and the sugar concn. was 15–100 μg . in a final volume of 10 ml. Equal weights of hexoses give values of 100%, of polyhexoses, 110% (cf. Holden, Pirie & Tracey, 1950). No interference by protein (gelatin), glucosamine hydrochloride, or mannitol was detectable when 50 μg . of these substances were added to 50 μg . of glucose.

Reducing substances. These were determined by Nelson's

Table 1. *Composition of mycelium preparations (moisture free)*

Mycelium	Yield (mg./g. <i>M</i>)	Composition (mg./g. mycelium)			
		Ash	N	P	Carbohydrate (as polyhexose)
<i>M</i> —whole mycelium	—	38	31.5	7.0	510
<i>Ms</i> —extracted by organic solvents	700	34	35	7.6	640
<i>Msw</i> —extracted by organic solvents and by water	630	14	34.5	3.3	700

(1944) adaption of the Somogyi method, without the preliminary precipitation of protein.

Amino sugar estimations. These were made on hydrolysates freed from the HCl used by repeated distillation *in vacuo*. The method of Elson & Morgan (1933) was modified by Mr M. V. Tracey of this department according to Blix (1948) and Immers & Vasseur (1950). Amino sugar was also determined by distillation with saturated Na_2PO_4 solution plus sodium borate, and estimation of the NH_3 produced by nesslerization (Tracey, 1951).

Paper chromatography. One-dimensional, descending paper chromatography was used for qualitative and quantitative examination of the carbohydrate components (Flood, Hirst & Jones, 1948). The sugar spots were developed with either ammoniacal AgNO_3 (Partridge, 1948) or aniline phthalate in glacial acetic acid (Hough, Jones & Wadman, 1950).

Colloidal material. This was isolated by dialysis in a cellophan membrane into frequently changed distilled water.

EXPERIMENTS AND RESULTS

In general, estimations were carried out, not only on whole mycelium (*M*), but also on mycelium that had been exhaustively extracted with organic solvents (*Ms*) and then with cold and hot water followed by a further extraction with ethanol (*Msw*) (cf. Table 1). Whether or no extracted material interfered with the determinations could be recognized.

Organic solvent extraction. This removed 31% of the dry weight of *M*. In the preparation of *Msw*, further extraction with ethanol removed another 1% of the dry weight of *M*. A little more fat-soluble material was liberated by hydrolysis of *Msw* with N-HCl in a boiling-water bath for 2 hr. Amounting to 2.5% of the dry weight of *M*, it was extracted from the solid residue by boiling ether-ethanol mixture (1:1) and from the aqueous layer by ether. More severe conditions of hydrolysis or refluxing over a flame produce traces of black 'humins' material which is itself soluble in ethanol. A total of 34.5% of the dry weight of *M* is, therefore, actually or potentially extractable from the mycelium with organic solvents.

No attempt was made to prepare pure compounds from these extracts, with the exception that mannitol was readily isolated by extraction with water, and after recrystallization had m.p. 165° , unchanged when mixed with an authentic specimen. The hexaacetate had m.p. 120° , also unchanged on admixture with an authentic specimen.

The combined material extracted with organic solvents contained 22% of the total N of mycelium *M*, and 24% of the total P.

Water extraction (cf. Tables 2a and b). Mycelium *M* (1 g.) extracted four times with water (25 ml.) in a steam bath, lost 29% of its dry weight. On dialysis 15% of the dry matter of the extract was retained as colloidal material. The extraction of mycelium *Ms* yielded dry matter equivalent to

Table 2a. *Composition of water extract of mycelium (M)*

	Content		
	mg./g. dry matter	Percentage of total constituent in <i>M</i>	Percentage of each constituent in colloidal fraction
Nitrogen	35	32	19
Carbohydrate (as glucose)	220	10	36
Reducing substance (as glucose)	20	—	—

Table 2b. *Composition of water extract of solvent-extracted mycelium (Ms)*

	Content		
	mg./g. dry matter	Percentage of total constituent in <i>M</i>	Percentage of each constituent in colloidal fraction
Nitrogen	40	7	75
Phosphorus	55	45	20
Carbohydrate (as glucose)	330	3	100

Table 3. *α -Amino-acid N content of HCl hydrolysates of mycelium*

(Mycelium taken into 12N-HCl solution, was diluted with an equal volume of water, and heated in a sealed tube immersed in boiling water, for 20 hr. The HCl was removed by vacuum distillation.)

Mycelium	α -Amino-acid N (mg./g. N of mycelium)	α -Amino-acid N (mg./g. N of mycelium <i>M</i>)
<i>M</i> —whole mycelium	46	46
<i>Ms</i> —extracted by organic solvents	56	44
<i>Msw</i> —extracted by organic solvents and by water	58	40

only 5.5% of the dry weight of mycelium *M*. On dialysis, 50% of the dry matter of the extract was retained.

Protein

Results are summarized in Table 3. A protein concentrate which was free, as far as could be ascertained, from other nitrogenous material was prepared by extraction of *Ms* with cold 2N-NaOH, precipitation of the carbohydrate with an equal volume of ethanol and, after bringing to pH 7 with glacial acetic acid, removal of the bulk of the ethanol by

distillation *in vacuo*. The small amount of insoluble material was spun off and contained less than 2% N. Trichloroacetic acid solution (20%) was added to the filtrate to a final concentration of 5% and the precipitate centrifuged off and washed with ethanol. About 0.04 g. of a pale-brown powder containing 9% N was thus isolated from 1 g. of *Ms*. After acid hydrolysis 720 mg. α -amino-acid N/g. total N was found. The apparent amino-sugar content, determined by the Elson & Morgan method was less than 1%, while in the case of Tracey's (1951) method, the $\text{NH}_3\text{-N}$ (50 mg./g. total N) was identical with the N given off as ammonia on distillation with saturated sodium phosphate solution. How far this figure of 720 mg. α -amino-acid N/g. total protein N is representative of the mycelial protein as a whole is not known, but under the conditions of hydrolysis used it is fairly typical of other protein preparations examined.

Assuming, therefore, that the mycelial protein of *M* yields 440 mg. α -amino-acid N/g. total N, and this value represents 72% only of the total protein N, then about 60% of the total N of mycelium *M* must be present in protein, which, using the conventional conversion factor 6.25, corresponds to 12% of protein in mycelium *M*.

Nucleic acid

An estimation of nucleic acid N and P was made following the method of Schneider (1945). Trichloroacetic acid solution (12.5 ml. of 10%) was added to mycelium *Ms* or *Msw* (1 g.) which had been allowed to soak in water (5 ml.) at room temperature for 30 min., and the mixture stirred for 20 min. After filtering with suction through madapollam the residue was refluxed, first with ethanol, then with chloroform. The air-dried product was then extracted with 5% trichloroacetic acid (25 ml.), with the extraction tube standing in boiling water, for 15 min. After filtering and washing, first with 5% trichloroacetic acid and then with water, extract and washings were combined. The cold trichloroacetic acid removed little more N and P from *Ms* than did water, and the extract from *Msw* was very slight. With hot trichloroacetic acid the amount of P extracted amounted to 110 mg./g. of total P in *M*, and of N to 70–100 mg./g. of the total N in *M*. Accepting the values found for phosphorus extracted, and assuming that nucleic acid contains about 10% P, this leads to a value of 8.0 mg. of nucleic acid/g. mycelium *M*.

After hydrolysis of *Ms* with $\text{N-H}_2\text{SO}_4$ for 7 hr. in a sealed tube immersed in boiling water, ribose was detected in the hydrolysate by paper chromatography. Quantitative measurements using a known amount of xylose as standard showed the presence of about 1.0 mg. ribose/g. *M*.

Chitin

The presence of polyacetylglucosamine in the mycelium (10 g.) was shown by hydrolysis with 6N-HCl at 100° for 6 hr. of the fraction of the mycelium remaining after ex-

traction with 2N-NaOH. Following distillation of the acid *in vacuo*, glucosamine hydrochloride was isolated from the solid residue by rubbing with acetone, followed by two crystallizations from aqueous ethanol. The colourless crystals (0.3 g.) had $[\alpha]_D^{20} + 71^\circ$ (c, 1.5 in water), after standing. An authentic sample of glucosamine hydrochloride gave $[\alpha]_D^{20} + 72^\circ$ (c, 1.5 in water), after standing. (Found N, 6.6. $\text{C}_6\text{H}_{13}\text{O}_5\text{NCl}$ requires N, 6.5%.) The osazone was identical in crystalline form with glucosazone prepared from an authentic specimen of glucosamine hydrochloride, and had m.p. and mixed m.p. 204–205°.

A mixture of the mycelium (10 g.) with 72% (w/w) H_2SO_4 (20 ml.) after standing for 2 days was diluted to 700 ml. and refluxed for 10 hr. The acid was partially neutralized with $\text{Mg}(\text{OH})_2$ and the product steam distilled. A definite positive test for acetic acid with lanthanum chloride and iodine (Krüger & Tschirch, 1930) was not given by this distillate, but when volatile reducing substances were fixed by oxidation with KMnO_4 beforehand, the steam distillate then gave a positive test. This effect is presumed to be due to the sulphur dioxide present in the hydrolysate, since sulphites were shown to reduce the sensitivity of the colour reaction. A small amount of a colourless, crystalline, water-insoluble material, which has not been identified, was filtered from the main bulk of the distillate which, after neutralization with NaOH was evaporated to small volume, acidified with HCl and extracted with ether. After evaporation of the ether, the extract smelt strongly of acetic acid and gave a *p*-phenylphenacyl ester, which after recrystallization had m.p. 109°. An authentic sample prepared from acetic acid had m.p. 110° and the mixed m.p. was 109°.

Adapting the method used by Black & Schwartz (1950) for the estimation of chitin in crawfish waste, mycelium (1 g.) was extracted once with cold 5% (w/v) NaOH (25 ml.) by standing, with frequent stirring, for 30 min. The insoluble material, spun down or filtered through madapollam under suction was extracted three times with the beaker standing in boiling water. Further extraction removed only traces of N. The residue was washed with water, *N*-acetic acid, again with water, refluxed with ethanol, followed by acetone, then chloroform, and air dried. Table 4 gives yields and analyses of the product, *M.alk.* The cold alkaline extract was used as a source of alkali-soluble carbohydrate.

After hydrolysis of *M.alk.* or chitin itself with 6N-HCl for 16 hr., no α -amino-acid N was found. Assuming, therefore, that the N content of *M.alk.* is entirely derived from amino-sugar residues, a minimum of about 9% of the total N of mycelium *M* is present in this form. This corresponds to about 19% of chitin in *M.alk.* and to about 4% in mycelium *M*.

Hydrolysis of mycelium with HCl, removal of the acid by distillation *in vacuo*, and estimation of amino sugar, gave further information. Initial experiments were carried out on an authentic sample of chitin (100 mg.) brought into 12N-HCl solution (1 ml.) by standing at room temperature, then

Table 4. Alkali-resistant residue *M.alk.*

Mycelium source	Yield (mg./g. of mycelium source)	Yield (mg./g. of mycelium <i>M</i>)	Nitrogen content	
			(mg. N/g. <i>M.alk.</i>)	mg. N in <i>M.alk.</i> /g. N in mycelium <i>M</i>
<i>M</i> —whole mycelium	230	230	13	84
<i>Ms</i> —extracted by organic solvents	320	220	13	90

The above figures refer to moisture-free material

diluting and heating in a sealed tube in boiling water. Amino-sugar content was estimated by Tracey's (1951) method. Heating with 6*N*-HCl for 3–15 hr. gave values between 86 and 92 % of the total N given off as NH₃, without a distinct maximum; 4*N*-HCl for 10 hr. gave about 75 % of the total N as NH₃, and 3*N*-HCl for 10 hr. gave about 70 %. Irving (1909), on polarimetric evidence, claimed that chitin was completely hydrolysed by conc. HCl in 8–10 hr. at 40–45°. However, chitin hydrolysed with 10*N*- or 12*N*-HCl for 10 hr. at 37° gave only 40 % of its N as NH₃ when distilled with sodium phosphate. Even after 48 hr. hydrolysis, no more than 50 % was obtained.

On the basis of these results, hydrolysis of mycelium was carried out by taking up the sample (100 mg.) in 12*N*-HCl (1 ml.), diluting after 2–3 days (when the mycelium was almost completely dispersed) with an equal volume of water and heating for 6 hr. in a sealed tube in boiling water. A small amount of black, insoluble material was formed, and care was taken to carry out estimations by the Elson & Morgan method on the clear, pale yellow or brown supernatant liquid.

In the case of mycelium hydrolysates the N volatile on distillation with sodium phosphate solution includes a proportion of NH₃-N derived from the degradation of amino-acids and this must be allowed for. However (Table 5) about 20 % of chitin N is also degraded to NH₃ and hence the net amounts of N in column 3 are taken to represent only 80 % of the total chitin N. A correction is made for this in column 4. About 12 % of the total N of mycelium *M* occurs in amino-sugar residues, corresponding to 5.5 % chitin in the mycelium.

Discordant results are obtained unless the laboratory atmosphere is clear. The size of the blanks are reduced if the steam source is made alkaline. Under these conditions the following results were obtained:

N-Acetylglucosamine. (Found: CH₃CO, 19.3. Calc. for C₈H₁₂O₅N.COCH₃: CH₃CO, 19.4 %.)

A chitin sample. (Found: N, 6.0; CH₃CO, 19.2. Calc. for anhydroacetylglucosamine, C₈H₁₀O₄N.COCH₃: N, 6.9; CH₃CO, 21.2 %.)

M.alk. (Found: CH₃CO, 4.1. Calc. for a chitin content of 19 %: CH₃CO, 4.0 %.)

Ms. (Found: CH₃CO, 1.7, corresponding to 1.2 % CH₃CO in mycelium *M*.)

Msw. (Found: CH₃CO, 1.75, corresponding to 1.1 % CH₃CO in mycelium *M*.)

M. (Found: CH₃CO, 1.5 %.)

If mycelium *M* contains 5.5 % of chitin, it would have 1.2 % CH₃CO. The higher percentage found in *M* itself must be due to the presence of acetyl compounds or other materials giving rise to volatile acid on hydrolysis, among the fat-soluble components. No higher fatty acids than acetic were detected in the distillate by paper chromatography using the method of Brown (1950). The titration figure was not reduced by the addition of KMnO₄ before distillation, confirming the absence of formic acid.

Carbohydrate

To estimate the carbohydrate content of mycelium by the orcinol method (Pirie, 1936), which obviates interference from protein or amino-sugar, the sample is taken up in 72 % (w/w) H₂SO₄ (4–6 mg./ml.) by allowing to stand at room

Table 5. *Glucosamine N in hydrolysates of mycelium*

(Hydrolysis by 6*N*-HCl for 6 hr. Results as mg./g. total N in material except in last two columns.)

Material	Ammonia N (AN)	Distilled N (DN)	DN-AN	(DN-AN) × 1.25	N (E & M)	Total N in mycelium <i>M</i> (mg./g.)	
						(DN-AN) × 1.25	N (E & M)
Glucosamine HCl	50–60	960–1050	900–1000	—	96–106	—	—
Chitin	70–80	870–920	780–850	—	93–110	—	—
Mycelium <i>M</i>	80–90	180–190	90–110	120	90–110	120	100
Mycelium <i>Ms</i>	60–70	190	120–130	150	130–140	117	100
Mycelium <i>Msw</i>	50–70	190–220	140	175	180	122	125
<i>M.alk.</i>	30–60	800–850	790–820	1000	720–870	—	—

N (E & M) refers to glucosamine N estimated by Elson & Morgan's (1933) method.

Distilled N refers to glucosamine N estimated by distillation with saturated sodium phosphate (Tracey, 1951).

Estimations of acetyl content were made by allowing the mycelium (50 mg.) to stand for several days in 72 % (w/w) H₂SO₄ (0.5 ml.), diluting to 12.5 ml. (to give an approximately *N*-acid solution) and heating in a boiling-water bath for 3 hr. After unsealing, the tubes were allowed to stand uncorked for about an hour to free the solution from volatile inorganic acid. No acetic acid is lost. 1 ml. of the solution, mixed with about 4 g. MgSO₄·7H₂O (to accelerate the distillation of the acid; Olmsted, Whitaker & Duden, 1929), was distilled in a Markham (1942) still at the rate of about 1 drop/sec., until exactly 10 ml. of distillate had been collected. The distillates were titrated with 0.2*N*-Ba(OH)₂ solution from a Conway microburette. Blank distillations were made from 1 ml. *N*-H₂SO₄ and 4 g. MgSO₄·7H₂O.

temperature for 1–3 days, when the solution from fat-free samples is completely clear and yellow to brown in colour. Mycelium *M* gives an opalescent solution due to acid-insoluble fatty components. The estimation is carried out at a suitable dilution. The values obtained were unaffected by the time the mycelium was allowed to stand in acid, up to 7 days.

The polyhexose content of *M* is taken as 45 %, calculated from the value obtained for *Ms* (Table 1). A certain amount of orcinol-positive material is therefore extracted by organic solvents, and this is presumed to be identical with the dialysable carbohydrate extracted by water from *M* (Table 2*a*). Although this water extract contains a small amount of reducing substance, no reducing sugar was

detected in it or the ethanolic extract by paper chromatography. After hydrolysis of a sample of ethanolic extract with $N-H_2SO_4$ glucose was detected by paper chromatography.

Only a small amount of colloidal carbohydrate is extracted by water from *M* or *Ms* (Tables 2a and b), but larger amounts are dispersed by cold 2*N*-alkali. By precipitation of the alkaline solution with an equal volume of ethanol (Norman & Peterson, 1932) a predominantly carbohydrate material is obtained representing 10–15% by weight of *M* and containing only 0.6–1.0% N. On hydrolysis with $N-H_2SO_4$ for 10 hr. in a sealed tube in boiling water, it was not taken entirely into solution, which contained only 600 mg. of reducing sugar (as glucose)/g. polysaccharide. On hydrolysis for 20 hr., after preliminary solution in 72% (w/w) H_2SO_4 , 850 mg. reducing sugar/g. of polysaccharide were obtained.

The alkali-extracted mycelium, *M.alk.*, contains about 74% of carbohydrate (as polyhexose), associated with chitin.

Ms, taken up in 72% (w/w) H_2SO_4 by allowing to stand for 2 days, diluted to give an *N*-acid solution, and hydrolysed for 20 hr. gave 73% of its dry weight as reducing substance, calculated as glucose. Paper chromatography indicated the presence of glucose, galactose, and mannose, approximately in the ratio 10:1.7:1, and these three sugars were also present in *M.alk.*, whereas the alkali-soluble polysaccharide contained only glucose. An osazone was prepared from a hydrochloric acid hydrolysate of this material (after removal of acidic and basic compounds with resin columns), identical in crystalline form, melting point and mixed melting point with an authentic specimen of glucosazone. Under conditions which readily yielded cellobiose octaacetate from cotton-wool cellulose, no such compound could be isolated from this alkali-soluble glucose polymer.

The presence of galactose in the mycelium was confirmed by oxidation with HNO_3 of an HCl hydrolysate of *Ms* (3 g.) freed from acidic and basic material, and isolation of mucic acid, m.p. 211–212° (decomp.); mixed melting point with an authentic specimen 212–213° (decomp.).

DISCUSSION

The preceding results lead to the figures for the composition of the mycelium given in Table 6. There is no indication that the mycelium contains 'lignin-like substances', and it is quite erroneous to suppose (as did Pinck & Allison, 1944) that the mycelium fraction insoluble after standing for 2 hr. in 72% (w/w) H_2SO_4 followed by refluxing after dilution to 3%, can be characterized as crude lignin. Even *M.alk.*, which certainly after alkali extraction contains no lignin, requires at least 24 hr. to disperse completely. On dilution of the clear solution and refluxing, a small amount of black material is formed but this is an invariable artifact

in the hydrolysis of material containing protein or amino sugar and carbohydrate.

Table 6. *Composition of mycelium M (moisture-free)*

(The air-dried mycelium contains in addition, about 10% moisture.)	
	mg./g. <i>M</i>
Fat and organic solvent-soluble material	345
Residual, dialysable, water-soluble material	30
Polyhexose carbohydrate	450
Protein	120
Chitin	55
Nucleic acid	10
Residual, unextracted ash	10
Total	1020 mg.

Nitrogen is distributed among the organic solvent extract (22%), residual water-extractable and dialysable material (5%), protein (60%), chitin (12%), and nucleic acid (4%).

About 70% of the total phosphorus is extracted by organic solvents and water and 10% remains in *M.alk.* (which surprisingly contains 0.3% P). Nearly 10% is present in nucleic acid.

The composition of *M.alk.* approximates to: polyhexose 74%; chitin 19%; ash, 1%. The remainder has not been accounted for.

SUMMARY

1. The composition of a sample of mycelium of *Penicillium griseofulvum* has been studied with particular reference to the high molecular weight components.

2. Fats and compounds soluble in organic solvents amount to about 35%.

3. Protein (about 12%) was estimated by a determination of α -amino-acid nitrogen on various fractions hydrolysed with hydrochloric acid.

4. Carbohydrate (about 50%) occurs for the most part as polyhexose. Glucose is the predominant sugar residue, but galactose and probably mannose are also present in the polysaccharide.

5. The presence of chitin (about 5.5%) was confirmed, but it was only isolated associated with carbohydrate.

6. There is no indication of the presence of lignin.

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The Component Acids of the Fats of some Indian Fresh-water Fish

By S. P. PATHAK AND C. V. AGARWAL

Department of Industrial Chemistry, Benares Hindu University

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The fats studied were from the body, viscera and liver of bhakur fish (*Catla buehanani*) and from the liver of pahuna fish (*Wallago attu*). These fish were from the River Ganges at Benares.

METHODS

The separated tissues (body, viscera or liver) were cut up into small pieces and extracted exhaustively with hot acetone. The extracted material was then taken up in light petroleum (b.p. 40–60°), the watery portion separated and the lipids recovered and dried at 100° under reduced pressure. The body and liver tissues were also treated by alkali digestion as recommended by Rapson, Schwartz & Van Rensburg (1943), but little fat could be extracted by this treatment. The fats were kept in 10 vol. of acetone at 0° to separate the phosphatides, if present, from the glycerides.

The fats were hydrolysed, and the mixed fatty acids recovered were resolved into groups of simpler mixtures of acids by the lithium salt-acetone (Tsujimoto, 1920; Tsujimoto & Kimura, 1923) and lead salt-ethanol methods. Each group of the acids was separately converted into methyl

esters, taking precautions recommended by Bjarnason & Meara (1944), and fractionated through an electrically heated and packed column. From the iodine values and equivalents of the ester fractions the composition of the latter were calculated by the method of Hilditch (1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturation, e.g. –2.0 (mono-ethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each group from which the mean equivalent of each of the homologous ester groups (C_{16} , C_{18} , C_{20} , C_{22} and C_{24}) follows. From the composition of each of the separated groups of fatty acids, that of the original fat was obtained.

RESULTS

The lipids and phosphatide contents of the tissues and the iodine values of the fats are given in Table 1.

Bhakur fats. The mixed fatty acids obtained by the hydrolysis of fats, as described earlier, from body (159.6 g.), viscera (202.8 g.) and liver (29.3 g.) were

Table 1. *Components of tissues of bhakur and pahuna*

	Bhakur			Pahuna liver
	Body	Viscera	Liver	
Water content (% on wet tissues)	67.5	40.2	72.7	68.3
Lipid content (% on wet tissues)	6.3	44.8	8.5	5.1
Lipid content (% on dry tissues)	19.0	75.0	31.3	16.1
Phosphatides in lipids (%)	0	0	5.4	12.5
Iodine value of the fat	97.9	87.2	90.5	122.9

Table 2. *Fractionation of tissue fats of bhakur*

Group description	Body		Viscera		Liver	
	Weight (%)	Iodine value	Weight (%)	Iodine value	Weight (%)	Iodine value
A Lead salt ethanol-insoluble	31.8	1.4	36.8	5.3	32.8	22.4
B Lithium salt acetone-insoluble but lead salt ethanol-soluble	47.8	111.8	49.9	105.0	67.2	138.0
C Lithium salt acetone-soluble	20.4	248.2	13.3	237.6	—	—

Table 3. *Fractionation of methyl esters of
bhakur body-fat acids A, B and C*

Fraction	Weight (g.)	Saponification equivalent	Iodine value
Methyl esters of acids A			
A1	3.05	254.6	0.2
A2	3.29	265.7	0.4
A3	3.86	268.0	0.6
A4	3.93	270.7	0.6
A5	4.01	271.2	0.6
A6	4.80	271.5	1.1
A7	3.26	272.2	1.1
A8	3.67	274.8	1.6
A9	5.37	284.3	2.4
A10	4.24	289.2*	11.6
	39.48		

Methyl esters of acids B			
B1	2.38	253.7	51.6
B2	3.16	267.1	72.0
B3	4.53	270.0	83.1
B4	4.97	272.7	83.8
B5	5.22	273.3	90.1
B6	3.57	273.8	93.0
B7	4.65	277.6	93.6
B8	4.75	278.0	99.9
B9	2.33	278.5	100.2
B10	5.02	278.7	102.6
B11	3.68	279.4	107.4
B12	4.20	281.9	112.3
B13	4.46	285.9	134.8
B14	2.04	299.6	169.7
B15	4.25	327.3*	183.1
	59.66		

Methyl esters of acids C			
C1	1.38	271.5	128.5
C2	2.84	281.2	153.3
C3	2.66	299.3	176.0
C4	3.67	308.4	256.7
C5	2.95	325.5	292.0
C6	3.05	335.6	332.7
C7	5.38	344.8	336.7
C8	3.90	345.8	340.9
C9	2.98	358.0*	143.6
	28.81		

* Equivalents of esters (freed from unsaponifiable matter): A10, 285.5; B15, 317.6; C9, 350.1.

separated into simpler groups of acids by the methods indicated in Table 2.

Each group of the acids was separately converted into methyl esters, which were fractionally distilled under reduced pressure through the column mentioned. The fractionation data for the methyl esters of the three groups of the body fat are summarized in Table 3.

The percentage composition, calculated from iodine values, and the equivalents and the mean unsaturation determined for each group of acids, and thence the component acids of the body fat, are recorded in Table 4.

Similarly, the component acids of the visceral and liver fats of bhakur were studied and their fatty acid compositions are given Tables 5 and 6, respectively.

Pahuna liver fat. 37.5 g. of the lipid, obtained in the usual manner from 736 g. of wet tissues, deposited 4.7 g. of crude phosphatides from a 10 % acetone solution at 0°. As the quantity of the acids available was small it was decided, unlike the bhakur liver fatty acids, to convert them as such into methyl esters and fractionate. The fractionation data are given in Table 7 and the figures for component fatty acids in the whole fat in Table 8.

DISCUSSION

Bhakur body, viscera and liver fats. It is unfortunate that not much work has so far been done on the fats of fresh-water fish, specially on those of Indian origin. Analyses of fats from different parts of the same animal have rarely been recorded, but such data as are available indicate that significant differences in composition may occur. This view is confirmed by the present studies.

In Tables 4-6 are given the component acids of bhakur body, viscera and liver fats. The saturated acid contents of body, viscera and liver fats are about 38, 37 and 30 %, respectively, and the contents of

the individual acids are somewhat similar—the chief difference being the much lower content of palmitic acid in the liver fat. It is also interesting to note the gradual increase in the relative proportions of saturated acids of higher molecular weights from body to viscera and to liver fats. The differences

(25 %), of viscera C_{18} (37 %) and of liver C_{20} (26 %), although the C_{18} acid content (25 %) of the liver fat is also almost equally high. Unsaturated C_{14} and C_{24} acids are minor components of these fats. In the visceral and the liver fats the unsaturated C_{16} acid group is much lower than in the body fat, but these

Table 4. *Component acids of bhakur body fat*

Acids	A (31.8 %)	B (47.8 %)	C (20.4 %)	Total	Whole fat excluding unsaponifiable	
					(%, w/w)	Moles %
Myristic	1.8	1.1	—	2.9	2.9	3.5
Palmitic	24.0	4.9	—	28.9	29.0	30.6
Stearic	5.2	1.3	—	6.5	6.5	6.2
Unsaturated:						
C_{14}	Trace	0.2	—	0.2	0.2	0.2
C_{16}	0.4	23.2	1.7	25.3	25.3	26.9
C_{18}	0.4	13.7	3.8	17.9	17.9	17.3
C_{20}	—	3.3	4.2	7.5	7.5	6.6
C_{22}	—	—	10.1	10.1	10.1	8.3
C_{24}	—	—	0.5	0.5	0.6	0.4
Unsaponifiable	Trace	0.1	0.1	0.2	—	—
Mean unsaturation of:						
C_{14}	-2.0	-2.0	-2.0	-2.0	—	—
C_{16}	-2.0	-2.0	-4.0	-2.7	—	—
C_{18}	-2.0	-2.3	-5.0	-2.5	—	—
C_{20}	-2.0	-5.0	-8.0	-5.7	—	—
C_{22}	—	—	-9.0	-9.0	—	—

Table 5. *Component acids in groups A, B, C and in the whole bhakur visceral fat*

Acids	Component acids in groups			Component acids in whole fat	
	A (36.8 %)	B (49.9 %)	C (13.3 %)	(%, w/w)	Moles %
Myristic	1.3	1.5	—	2.8	3.4
Palmitic	24.7	0.6	—	25.3	26.9
Stearic	7.4	0.5	—	7.9	7.6
Arachidic	1.4	—	—	1.4	1.2
Unsaturated:					
C_{14}	Trace	0.4	1.0	1.4	1.6
C_{16}	0.7	6.0	3.3	10.0	10.8
C_{18}	0.7	33.2	3.3	37.2	36.1
C_{20}	0.6	7.7	3.6	11.9	10.6
C_{22}	—	—	2.1	2.1	1.8
Unsaponifiable	Trace	Trace	Trace	—	—
Mean unsaturation of:					
C_{14}	-2.0	-2.0	-2.0	-2.0	—
C_{16}	-2.0	-2.0	-4.0	-2.7	—
C_{18}	-2.0	-2.3	-5.0	-2.5	—
C_{20}	-2.0	-5.0	-8.0	-5.7	—
C_{22}	—	—	-9.0	-9.0	—

become marked in the unsaturated portions. Here also, in a general way, the relative proportions of acids of higher molecular weights increase in the same order accompanied by a general decrease in the mean unsaturation—the mean unsaturation of C_{16} and C_{18} acids in the visceral fat deviating slightly from the rule. In the unsaturated acid portions the chief component acid in the fat of body is C_{16}

fats are richer in unsaturated C_{18} acids. Liver fat is rich in C_{20} acids. Visceral fat has a small amount of C_{22} acids, but body and liver fats have larger and almost equal amounts (10 %) of this group.

The analyses show large differences in the contents of the fatty acids in the body, visceral and liver fats of bhakur, specially in the unsaturated acid portions. These results are somewhat different from

those for jacobever (*Sebastichthys capensis*) (Van Rensburg, Rapson & Schwartz, 1945), inasmuch as that the jacobever visceral fat resembles the body fat; but in the liver fat the proportion of C_{18} acids is

differences in the unsaturated acids of the body and liver fats of turbot and halibut. Hilditch & Pathak (1949) found that blubber and liver fats of the common seal showed distinct differences in their

Table 6. *Component acids of bhakur liver fat*

Acids	A (32.8%)	B (67.2%)	Total	Acids excluding unsaponifiable	
				(%, w/w)	Moles %
Myristic	0.6	—	0.6	0.6	0.7
Palmitic	16.5	2.4	18.9	19.0	21.3
Stearic	4.1	2.3	6.4	6.4	6.5
Arachidic	3.1	—	3.1	3.1	2.9
Unsaturated:					
C_{14}	Trace	—	Trace	Trace	Trace
C_{16}	1.4	5.5	6.9	7.0	7.9
C_{18}	0.9	24.3	25.2	25.4	25.8
C_{20}	5.8	20.5	26.3	26.5	24.8
C_{22}	—	9.3	9.3	9.4	8.1
C_{24}	—	2.5	2.5	2.6	2.0
Unsaponifiable	0.4	0.4	0.8	—	—
Mean unsaturation of:					
C_{16}	-2.0	-2.0	-2.0	—	—
C_{18}	-2.0	-2.7	-2.7	—	—
C_{20}	-2.0	-5.1	-4.8	—	—
C_{22}	—	-6.5?	-6.5?	—	—
C_{24}	—	-8.0?	-8.0?	—	—

Table 7. *Fractionation data of methyl esters of pahuna liver fat acids*

Fraction no.	Weight (g.)	Saponification equivalent	Iodine value
1	1.82	262.0	27.9
2	2.07	270.3	28.9
3	1.84	278.0	41.1
4	2.07	288.7	79.1
5	2.37	293.6	92.7
6	2.05	295.6	98.9
7	2.37	301.3	120.0
8	2.27	318.6	207.6
9	1.97	333.7	259.0
10	2.10	338.3	283.2
11	2.32	353.7*	104.9

* Equivalent of ester (freed from unsaponifiable matter): 327.0.

Table 8. *Component acids of pahuna liver fat*

Acids	%	(%, w/w)	Moles %
Myristic	1.44	1.5	1.8
Palmitic	14.15	14.2	15.9
Stearic	4.00	4.0	4.1
Unsaturated: C_{14}	0.42 (-2.0)	0.4	0.5
C_{16}	8.42 (-2.0)	8.4	9.5
C_{18}	32.45 (-2.4)	32.6	33.2
C_{20}	19.76 (-5.3)	19.8	18.5
C_{22}	19.00 (-8.0)	19.1	16.5
Unsaponifiable	0.36	—	—

about 46 as compared with 28% for the body whilst the C_{20} and C_{22} acids are 13 and 8% as compared with 22 and 17% for the body fat. Other small noticeable differences (of 2-3%) in the body and the liver fats are in palmitic, stearic and unsaturated C_{21} acids. Lovern (1932, 1937) found similar

saturated acids and still greater differences in their unsaturated acids.

Unfortunately not many visceral fats have been studied so far. But some of the analyses of visceral fats have been made in cases in which data is also available for the blubber fat of the same animal, even if it has not been studied by the same person. The composition of herring visceral fat found by Hilditch & Pathak (1948) is widely different from that of herring body fat given by Lovern (1938) with respect to unsaturated acids and there are smaller differences in the saturated acids. The ling visceral and liver fat analyses of Shorland (1939) show specific differences in their C_{18} , C_{20} and C_{22} acids.

Pahuna liver fat. In comparison with bhakur, the pahuna has more phosphatide in the liver lipids, and the liver fat is less rich in saturated acids. Palmitic acid is the chief saturated acid in both. The C_{16} unsaturated acid content is similar. The chief unsaturated components of pahuna liver fat are C_{18} unsaturated acids (33%, which is higher than in the bhakur), but the C_{20} acid group (20%) and C_{22} acid group are major components. Comparison with published figures for fats of fresh-water fish from other parts of the world gives the impression that the Indian fish livers are low in C_{16} and high in C_{22} unsaturated acids, but the comparison may not be valid, for the published figures are generally not for liver fats, known to be richer in acids of higher molecular weight.

It is worth while to draw attention to the potential source of fat for industrial purposes in the viscera of

bhakur. The wet tissue, now discarded, contains 45% of fat, the recovery of which should be remunerative.

SUMMARY

1. The compositions of body, viscera and liver lipids from bhakur (*Catla buehanani*), and of liver fat of pahuna (*Wallago attu*) have been studied. Preliminary separations of the fatty acids into groups differing in unsaturation have been carried out with the help of lithium and lead salts in acetone and ethanol, respectively, and the compositions studied by the ester fractionation procedure.

2. The component acids of bhakur body, viscera and liver fats show significant differences in their proportions and degree of unsaturation. The fat content of viscera is remarkably high, 45%, as compared to 6.3% in the body and 8.5% in the liver on wet tissues, and industrial exploitation of the fat has been suggested.

3. The liver lipids of pahuna contain more phosphatides than bhakur liver fat, and there are minor differences in the acid components.

We are glad to express our most cordial thanks to Prof. T. P. Hilditch, F.R.S., for his very useful suggestions and kind help during the progress of this work.

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The Formation of Hydrogen Peroxide by α -Radiation in the Presence of the Enzyme Carboxypeptidase

By W. M. DALE, J. V. DAVIES, C. W. GILBERT AND J. P. KEENE
Christie Hospital and Holt Radium Institute, Manchester

AND L. H. GRAY

Radiotherapeutic Research Unit, Medical Research Council, Hammersmith, London, W. 12

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The enzyme carboxypeptidase is inactivated by exposure to either α -radiation or X-radiation in aqueous solution. In all except the most concentrated solutions, the inactivation is due almost entirely to energy dissipated as ionization and excitation, in the water, leading to the formation of radicals or other active chemical intermediates which enter into the reaction with the enzyme. Whilst this is true of both types of radiation, the number of enzyme molecules inactivated per unit energy dissipated in the water by α -radiation is only about one-twentieth of the number inactivated by an equal dose of X-radiation. When these experimental observations were reported (Dale, Gray & Meredith, 1949), it was pointed out that the small amount of inactivation brought about by α -radiation could, in fact, be reasonably ascribed to the very low energy electrons, or δ -rays, which branch off as spurs along an α -particle track, in which case it would

appear that the main part of the α -ray energy which is dissipated within a narrow column is almost without effect on the enzyme. It was natural to associate the absence of effect on the enzyme with the well established fact that α -ray energy, unlike X-ray energy, gives rise to hydrogen peroxide and hydrogen when dissipated in pure air-free water, and to suppose that at all enzyme concentrations used in our experiments the radicals formed at very high concentration within the α -ray column combine together to form hydrogen peroxide and hydrogen before diffusion has brought them into effective collision with enzyme molecules. In this case, the presence of the enzyme should have little or no effect on the hydrogen peroxide yield, since the enzyme does not react with hydrogen peroxide. An alternative interpretation might be to assume that, owing to the very high concentration of radicals, interaction with enzyme molecules is confined to

those few molecules lying close to the track, so that many radicals in succession give up their energies to a few already inactivated enzyme molecules. The presence of enzyme in this case should cause a decrease in hydrogen peroxide yield. The fact that carboxypeptidase activity is hardly affected by high concentrations of hydrogen peroxide has made possible a comparison of the hydrogen peroxide yield by α -radiation in water and in a solution of carboxypeptidase. The results show that the alternative mechanism has to be rejected.

EXPERIMENTAL

The experimental technique followed closely that of former experiments (Dale *et al.* 1949). Radon was divided approximately equally between, and sealed into, two small and thin glass bulbs. These were then broken within a few minutes of pumping, one in a gas-tight vessel completely filled with water, and one in a similar vessel filled with the carboxypeptidase solution. The amount of radon in each solution was measured by following the rise of the γ -activity of the solutions, using a conventional ionization chamber and electrometer valve circuit. An irradiation of

tion of H_2O_2 was carried out colorimetrically as its titanium compound with a Hilger absorptiometer.

The concentration of the enzyme solution was 0.1% (w/v). In order to render the H_2O_2 formed by α -radiation less liable to decomposition, the enzyme solution and the control were adjusted to pH 3.8 by addition of H_2SO_4 . This pH is on the acid side of the pH of minimum solubility and of the isoelectric point of the enzyme. All solutions contained O_2 in equilibrium with air.

Control experiments were made to determine the loss of water by evaporation during the blowing off of the radon.

RESULTS

The results of the experiments are set out in Table 1. As in the earlier experiments, the radiation received by the solution can be divided into three parts: (1) Radiation from active deposits laid down on the splinters of the glass bulb between the time of pumping the radon and breaking the bulb (half of this radiation is assumed to be absorbed by the splinters). (2) Radiation from radon and its active deposit during the time the radon is in solution. (3) Radiation from the active deposits remaining in the solution after removal of the radon, and continuing up to the moment of making the optical density measurement.

Table 1. *Effect of carboxypeptidase on the hydrogen peroxide yield from water irradiated at pH 3.8 by α -rays*

(Significance test of difference of ionic yield gives $d/\sigma = 1.02$ and $P = 0.30$.)

	Carboxypeptidase	Water
Time of breaking bulb (t_1) (min.)	2.7	3.2
Time of removing radon (t_2) (min.)	107	114
Time of measurement (t_3) (min.)	250	262
Initial amount of radon (mc.)	61.2	60.0
Volume of irradiated solution (ml.)	3.07	2.85
Volume of solution, after evaporation, used in measurement (ml.)	2.34	0.66
Estimated loss of water by evaporation (ml.)	0.20	0.05
Amount of H_2O_2 measured ($\mu g.$)	103.5	29.5
Fraction of H_2O_2 measured	0.83	0.25
Amount of H_2O_2 in irradiated solution ($\mu g.$)	125.0	118.1
α -Energy liberated in solution (joules)	43.0	44.9
β -Ray correction:		
β -Energy liberated in solution (joules)	2.2	2.3
Estimated H_2O_2 due to β -radiation ($\mu g.$)	3.1	3.2
Amount of H_2O_2 due to α -radiation ($\mu g.$)	121.9	114.9
Ionic yield for α -radiation	0.264	0.239
Estimated errors (%) of:		
Absorption measurement	2.4	8.5
Fractionation of solution	2	3
Relative amounts of radon	1	1
Total error (%)	3.3	9.6

about 1.5 hr. was given, and an attempt made to compensate for a small difference in the distribution of radon between the two glass bulbs by adjusting the time of irradiation. The irradiation was ended by pouring the solutions into shallow dishes in the open air and assisting the removal of the radon by a jet of air which also agitated the solution. A further period of 2 hr. was allowed for the decay of most of the active deposits remaining in solution, and then the estima-

The α -particles come from the disintegration of radon and the active deposits of RaA , RaC and RaC' , and in calculating the energy delivered by them, account must be taken of the growth and decay of these products. This can most conveniently be done by calculating an auxiliary function, $F(t)$, which is the total energy delivered to a solution by the α -particles from radon and its products up to time t , after starting at time $t=0$ with radon free from active deposits.

It is convenient to normalize the function so that it has the value unity after full decay of the radon, and to use the fact that 1 mc. of radon going to full decay gives in α energy, from itself and its products, 54.09 joules. The function $F(t)$ has the form

$$F(t) = 1 - \sum_{r=1}^4 \gamma_r e^{-\lambda_r t}$$

with the following values for the constants λ and γ :

	1 (Rn)	2 (RaA)	3 (RaB)	4 (RaC)
λ	0.000126	0.227	0.0259	0.0352 min. ⁻¹
γ	+1.003800	-0.000179	-0.008369	+0.004745

It is then easy to show that the total energy delivered per mc. of radon to the experimental solutions, assuming half the radiation from the active deposits on the glass splinters to be absorbed by the glass, is $54.09 \times E$ joules, where

$$E = \frac{1}{2} [F(t_3) + e^{-\lambda_1 t_1} F(t_3 - t_1) - F(t_1)] - e^{-\lambda_1 t_2} F(t_3 - t_2)$$

and t_1 , t_2 and t_3 are the times in minutes after pumping the radon to breaking the bulb, removing the radon and estimating the H_2O_2 produced. In these calculations it is assumed that the decay of short-lived RaC' (half life about 10^{-6} sec.) follows instantaneously after that of the parent RaC, and that the radiations from the long lived deposits RaD, etc., can be neglected.

The solutions are, in addition to the α -rays, irradiated by the β -particles, and the energy contributed by these is about 5.2% of the α -energy. Assuming that the β -radiation acts independently of the α -radiation, the amount of H_2O_2 produced by the β -radiation has been estimated from the experiments of Bonet-Maury & Lefort (1950), due allowance being made for dose rate and the pH of the solution.

In calculating the ionic yield, the energy expended per ion-pair was taken as 32.5 eV., leading to 1.92×10^{17} ion-pairs per joule.

The errors of the different parts of the experiment have been estimated and are shown in Table 1. The difference of the ionic yield with and without the enzyme, when compared with the estimated error of the difference was not significant.

DISCUSSION

The results show that the addition of the enzyme carboxypeptidase does not reduce the yield of H_2O_2 formed by irradiation of water with α -particles. Thus the second hypothesis put forward in the introduction as an explanation of the low α efficiency for inactivation of the enzyme must be discarded and the result is consistent with the first hypothesis.

It will be seen from Table 1 that an allowance of approximately 3% has been made for the H_2O_2 produced by the β -radiation which accompanies the

α -radiation. It is difficult to evaluate this correction with accuracy because of the complexity of various competing reactions. The α -radiation alone gives rise to a final H_2O_2 concentration of around 1000 μ mole/l. which, according to Bonet-Maury & Lefort (1950), approaches the steady state concentration of H_2O_2 resulting from the exposure of aerated water to X-radiation and which presumably is also comparable with the steady state concentration resulting from exposure to β -radiation.

There is, therefore, some doubt as to whether the β -radiation has augmented or decreased the H_2O_2 concentration produced by the α -radiation in pure water. It is also clear that when the concentration of H_2O_2 is such that there is an approximate balance between its rate of production and rate of destruction by radicals formed by β -radiation, the introduction of a solute, such as carboxypeptidase, may disturb the balance in either direction on account of differences of affinity for the different types of radicals. The slightly greater yield of hydrogen peroxide observed by irradiating a carboxypeptidase solution as compared with that obtained from irradiated pure water, could, therefore, have arisen secondarily through the disturbing effect of the small amount of β -ray energy accompanying the α -radiation. As mentioned earlier in the text, however, the difference is not statistically significant.

In conclusion we can say that the experiments reported here confirm the view suggested in the earlier publication (Dale *et al.* 1949) that the production of H_2O_2 by α -radiation takes place in the core of the track and that the small amount of inactivation of carboxypeptidase by α -radiation is due to the very low energy electrons or δ -rays which branch off as spurs from the α -track.

SUMMARY

The yield of hydrogen peroxide produced by the action of α -rays from radon on water and on a 0.1% solution of the enzyme carboxypeptidase have been compared and no significant difference found. This result confirms the view suggested in an earlier publication that the production of hydrogen peroxide by α -radiation takes place in the core of the track and that the small amount of inactivation of carboxypeptidase by α -radiation is due to the δ -rays which branch off as spurs from the α -track.

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Studies in the Biochemistry of Micro-Organisms

88. PALITANTIN. PART 2. FURTHER DERIVATIVES AND DEGRADATION PRODUCTS

By J. H. BIRKINSHAW

Department of Biochemistry, London School of Hygiene and Tropical Medicine,
University of London

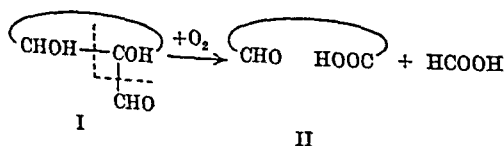
(Received 19 October 1951)

The isolation of the mould metabolic product, palitantin, $C_{14}H_{22}O_4$, obtained from *Penicillium palitans* Westling, was described by Birkinshaw & Raistrick (1936). The product was characterized as an aldehyde containing two hydroxyl groups and apparently possessing two double bonds. The function of the fourth oxygen atom was not determined. Further derivatives and degradation products of palitantin have been prepared and are described in the present communication.

In the earlier paper (Birkinshaw & Raistrick, 1936) palitantin was described as optically inactive, since under the experimental conditions employed the rotation observed, $+0.01^\circ$, was regarded as zero within the limits of experimental error. Some of the derivatives and breakdown products have now been found to show considerable optical activity. When palitantin was re-examined under more favourable conditions it was found to have a rotation of $[\alpha]_{D}^{25} + 4.4^\circ$ (*c.* 0.8 in $CHCl_3$).

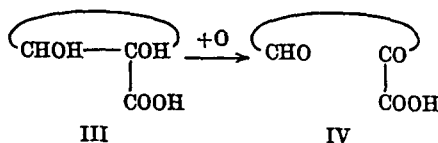
In a quantitative study of the periodate oxidation of palitantin, tetrahydropalitantin, and tetrahydropalitantic acid, the following points have been established. (1) Palitantin and tetrahydropalitantin react with potassium periodate using the equivalent of 2 atoms of oxygen. (2) Two acidic groups are produced, one of which readily lactonizes. (3) An aldehyde group is also produced. (4) The aldehyde and lactonic groupings are constituents of a C_{13} molecule, the other carbon atom being detached as formic acid. (5) With tetrahydropalitantic acid, only one oxygen atom is used, no acidic group is produced but two carbonyl groups are formed.

These facts appear capable of a simple explanation, namely that the reactive grouping in palitantin is part of a ring, and has the form I.



This would be split at the dotted lines by 2 molecules of periodic acid to yield form II plus 1 molecule of formic acid. With tetrahydropalitantic acid,

in which the aldehyde group of tetrahydropalitantin has been oxidized to carboxyl, the starting material would be represented by form III which on oxidation with 1 molecule of periodic acid would pass to IV.



Since the C_{13} acid lactone from tetrahydropalitantin gives rise on nitric acid oxidation to a *n*-heptyl succinic acid, tetrahydropalitantin would appear to consist of a 6-carbon ring with two side chains, one being a CHO group and the other a *n*-heptyl chain. One double bond in palitantin must be situated $\gamma\delta$ to the terminal methyl group of the C_7 chain to account for the production of *n*-butyraldehyde on ozonolysis. The position of the second double bond, if present, is still obscure, likewise the function of the fourth oxygen atom which is presumably attached to a ring carbon atom.

The examination of palitantin continues and it is hoped to discuss its constitution more fully in a later paper.

The dextrorotatory acid which was obtained, together with palitantin, as a metabolic product of the type strain of *Penicillium palitans* (Birkinshaw & Raistrick, 1936, p. 803), has been further examined. It crystallizes from water in colourless needles, m.p. 134.5° , $[\alpha]_{D}^{25} + 82^\circ$ (in $CHCl_3$). It has the empirical formula $C_{14}H_{20}O_4$, titrates as a monobasic acid and gives a reddish-purple colour with ferric chloride. Curtis, Hemming & Smith (1951) have recently isolated an antifungal metabolic product, frequentin, from a strain of *Penicillium frequentans* Westling, which was also found to produce palitantin. From the published description frequentin appeared to be very similar to the acid product from *P. palitans*. By a comparison of samples it has been established that the two products are in fact identical; there was no depression of melting point on mixing. The acidic product from *P. palitans* is

therefore frequentin. Curtis & Duncanson (1952) have now shown that frequentin and palitantin are closely related in structure.

EXPERIMENTAL

All melting points are uncorrected.

Properties of palitantin and its derivatives

Specific rotations. Birkinshaw & Raistrick (1936) stated that palitantin is optically inactive. This opinion was based on the fact that a solution of palitantin (0.1505 g.) in CHCl_3 (15 ml.) had an observed average rotation of $+0.01^\circ$ (limits of five readings 0.00° to $+0.02^\circ$) in a 10 cm. tube in the Hg green light (5461 Å.) at 20° . This corresponds to $[\alpha]_{5461}^{20} = +1.0^\circ$, which was considered to be within the limits of experimental error.

When it was subsequently observed that derivatives of palitantin have relatively high specific rotations, the specific rotation of palitantin was redetermined. A solution of palitantin in CHCl_3 was exhaustively extracted with aqueous alkali to remove traces of frequentin (see p. 276). The purified CHCl_3 solution was evaporated to dryness *in vacuo*, and the residual palitantin was crystallized a number of times from boiling water. A solution of the purified palitantin (0.3941 g.) in CHCl_3 (50 ml.) in a 40 cm. tube had an observed average rotation of $+0.14^\circ$ (limits of seven readings $+0.13^\circ$ to $+0.15^\circ$), corresponding to $[\alpha]_{5461}^{23} = +4.4^\circ$.

The observed specific rotations of palitantin and some of its derivatives are recorded in Table 1.

whole of the volatile acids had been removed in the distillate. The total volatile acid (10.23 ml. of 0.1N) corresponds to 1.003 mol. of acetic acid/mol. of palitantin.

Tetrahydropalitantin. Tetrahydropalitantin (0.2876 g.) on similar oxidation yielded 11.03 ml. 0.1N volatile acid, equivalent to 0.989 mol. of acetic acid per mol. of tetrahydropalitantin.

Reaction of palitantic acid with sodium amalgam and of palitantin with maleic anhydride

With sodium amalgam. Palitantic acid (1 g.) was treated with Na amalgam (2.5% Na; 100 g.) and water with vigorous stirring. The reaction was maintained about neutral by frequent additions of H_2SO_4 . The solution was filtered whilst slightly alkaline and acidified. Crystals (0.8 g.) were obtained, which proved to be unchanged palitantic acid. The reduction was therefore continued using a further 400 g. of amalgam over a period of 2 days. Unchanged palitantic acid (0.65 g.) was again recovered, an almost quantitative recovery when allowance is made for the solubility of palitantic acid. A further attempt to obtain reduction in acidic ethanol also failed. This renders unlikely the presence of a conjugated double bond or of a grouping capable of keto-enol tautomerism.

Action of maleic anhydride on palitantin. A solution of palitantin (0.2 g.) and maleic anhydride (0.1 g.) in benzene (100 ml.) was refluxed for 1.5 hr. On cooling, crystals separated and were collected, washed with benzene and dried, wt. 0.13 g., m.p. $164\text{--}165^\circ$ not depressed on admixture with palitantin. Hence palitantin does not appear to contain a conjugated double bond system.

Table 1. *Specific rotation of palitantin and its derivatives*

Substance	Temperature ($^\circ$)	Solvent	Vol. of solvent (ml.)	Wt. of substance (g.)	Length of tube (cm.)	$[\alpha]_{5461}^{23}$ ($^\circ$)
Palitantin	23	CHCl_3	50	0.3941	40	+ 4.4
Palitantin monophenyl- hydrazone	24	Pyridine	1	0.0088	5 microtube	- 93
Palitantin bis- <i>p</i> -bromo- benzoate	24	Ethanol	1	0.0110	5 microtube	- 13
Palitantic acid	20	Ethanol	1	0.0100	5 microtube	- 74
α -Hexahydropalitantin	24	Ethanol	1	0.0074	5 microtube	- 54
β -Hexahydropalitantin	24	Ethanol	1	0.0108	5 microtube	- 72
Tetrahydropalitantic acid	24	Ethanol	1	0.0102	5 microtube	- 90
Tetrahydropalitantin bis- <i>p</i> -bromobenzoate	23	Ethanol	25	0.0666	20	- 2
Tetrahydropalitantin	24	Ethanol	50	0.5836	40	- 43.1

Carbon-methyl groups

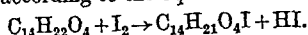
Palitantin. 1.19 and 1.18 mol. of acetic acid were produced per mol. of palitantin in the Kuhn-Roth micromethod. (Estimations by Dr Roth.)

A mixture of palitantin (0.2589 g.), CrO_3 (12 g.), $\text{K}_2\text{Cr}_2\text{O}_7$ (3 g.), water (30 ml.) and H_3PO_4 (sp.gr. 1.75, 20 ml.) was heated on a boiling-water bath under reflux for 3.5 hr. (Karrer, Helfenstein, Wehrli & Wettstein, 1930). A distinct smell of butyric acid was observed. The oxidation mixture, contained in a Claisen flask, was distilled *in vacuo* until the

Derivatives of palitantin, tetrahydropalitantin and palitantic acid

Monoiodopalitantin. 0.1N Aqueous I_2 solution (100 ml.) was added to a solution of palitantin (0.5 g.) in ethanol (25 ml.). The solution, from which crystals soon began to separate, was held for 1 hr. initially at room temperature and finally in the refrigerator. The excess I_2 was removed by titration with chilled 0.1N- $\text{Na}_2\text{S}_2\text{O}_3$. I_2 , equivalent to 40 ml. 0.1N, was absorbed, corresponding almost quantitatively to

the uptake of 2 atoms of I/mol. of palitantin. The slightly yellow crystals (0.47 g.) were collected, dried and recrystallized from aqueous ethanol (2 vol. water: 1 vol. ethanol). Beautiful long colourless silky needles (0.30 g.) of monoiodopalitantin separated, m.p. 136° (sharp, but decomposing to a black liquid which froths up the melting point tube). (Found: C, 44.5, 44.6; H, 5.7, 5.7; I, 33.7, 33.4. $C_{14}H_{21}O_4I$ requires: C, 44.2; H, 5.6; I, 33.4%). Monoiodopalitantin is thus derived according to the equation



Monoiodopalitantic acid. 0.1N Aqueous I_2 solution (20.0 ml.) was added to a solution of palitantic acid (0.0540 g.) in 0.1N aqueous NaOH (2.05 ml.), and the whole made up to a total volume of 50.0 ml. After 1 hr. the residual I_2 was estimated by titration with 0.1N- $Na_2S_2O_3$. Iodine equivalent to 1.95 atoms/mol. of palitantic acid was absorbed. The titrated solution was acidified with 0.1N-HCl (2 ml.). After chilling, colourless needles (0.023 g.) of monoiodopalitantic acid separated and were collected, washed and dried. Melting point 135.5°, darkening on melting, but not blackening or frothing as did monoiodopalitantin. (Found: C, 42.1, 42.3; H, 5.5, 5.6; I, 31.8, 31.5. $C_{14}H_{21}O_4I$ requires: C, 42.4; H, 5.3; I, 32.0%.)

Palitantin monoxide. When palitantin is treated with a $CHCl_3$ solution of camphoric acid peracid at room temperature (3.8 g. in 100 ml. $CHCl_3$ solution as determined by thiosulphate titration—Milas & Cliff, 1933), oxidation takes place corresponding roughly with the oxidation of one double bond in 1 hr. and two double bonds in 20 hr.

Palitantin (0.25 g.) was treated with an excess (25 ml.) of camphoric acid peracid solution in $CHCl_3$ and held for 1 hr. at room temperature. The palitantin gradually dissolved and had completely dissolved in less than 1 hr. The mixture was then thoroughly extracted with aqueous $NaHCO_3$. On removal of the solvent from the $CHCl_3$ layer, a colourless solid remained which was recrystallized from ether (50 ml.). Palitantin monoxide was thus obtained as beautiful colourless clusters of needles (0.03 g.), m.p. 144°. It is fairly readily soluble in water. (Found: C, 62.7, 62.9; H, 8.3, 8.3. $C_{14}H_{22}O_5$ requires: C, 62.2; H, 8.2%.)

A solution of palitantin (1 g.) in 100 ml. of a $CHCl_3$ solution of camphoric acid peracid was held at room temperature for 18 hr. The oxygen absorbed corresponded almost exactly to 2 atoms/mol. of palitantin. The oxidation product, isolated as described in the previous paragraph, proved to be mainly an uncrystallizable oil containing 0.06 g. of palitantin monoxide, m.p. 137°. (Found: C, 61.9, 61.8; H, 8.2, 8.0%. $C_{14}H_{22}O_5$ requires: C, 62.2; H, 8.2%.)

Tetrahydropalitantin bis-p-bromobenzoate. A mixture of tetrahydropalitantin (0.25 g.), p-bromobenzoyl chloride (0.8 g.) and pyridine (5 ml.) was heated on a boiling-water bath for 10 min. Crystals formed on cooling. Water was added and decanted after a time. The residue was thoroughly extracted with aqueous $NaHCO_3$ and finally with water. The sticky residue was boiled with ethanol (30 ml.) and the residual crystals were separated and identified as bromobenzoic anhydride. By the cautious addition of water to the ethanolic mother liquors a sticky first crop of crystals was obtained which was rejected. The second crop was crystalline and, after recrystallization from water, tetrahydropalitantin bis-p-bromobenzoate was obtained as colourless clusters of flattened needles, m.p. 94.5°. (Found: C, 53.9, 53.9; H, 5.2, 5.3; Br, 25.4. $C_{22}H_{22}O_8Br_2$ requires: C, 53.8; H, 5.2; Br, 25.6%.)

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Breakdown products and their derivatives

Hydrolysis of the dihydrazide $C_{13}H_{28}O_3N_4$ obtained by oxidation of tetrahydropalitantin with silver oxide

A solution of the dihydrazide (Birkinshaw & Raistrick, 1936, p. 807). (0.025 g., m.p. 190°) in 10N- H_2SO_4 (5 ml.) was boiled under reflux for 0.5 hr. After cooling, the hydrolysate was diluted with water (20 ml.) and thoroughly extracted with ether. On removal of the solvent and thorough drying *in vacuo*, a syrup (0.0185 g.) remained and showed no signs of crystallization. It was dissolved in ethanol (1 ml.) and titrated in the cold to phenolphthalein with 0.1N-NaOH; 0.81 ml. of 0.1N-NaOH were required, corresponding to an equivalent of 228. A mono-acid monolactone of the formula $C_{13}H_{22}O_4$, titrating as a monobasic acid, requires 242.

An excess (2.00 ml.) of 0.1N-NaOH was now added and the whole was heated on the water bath for 1 hr. After cooling, 1.27 ml. of 0.1N-HCl was required to neutralize to phenolphthalein. Hence the additional alkali used in the hydrolysis = 0.73 ml., giving a total amount of alkali used of 0.81 + 0.73 ml. = 1.54 ml. 0.1N. This corresponds to a total equivalent of 120. $C_{13}H_{22}O_4$, i.e. the mono-lactone of $C_{13}H_{24}O_5$, titrating as a dibasic acid requires an equivalent of 121.

Oxidative fission of palitantic acid. Formation of n-butyraldehyde

With $KMnO_4$. A solution of $KMnO_4$ (2 g.) in water (200 ml.) was added dropwise to a solution of palitantic acid (1 g.) in water (150 ml.) and N-NaOH (4 ml.). During the oxidation a brisk current of air was passed through the solution, and the issuing gas was scrubbed through bubblers containing Brady's reagent (2:4-dinitrophenylhydrazine in 2N-HCl). The resulting yellow precipitate was collected, dried and crystallized from methanol. Yellow needles, m.p. 123.5°, not depressed on admixture with authentic n-butyraldehyde 2:4-dinitrophenylhydrazone, m.p. 123°. (Found: C, 47.4, 47.4; H, 4.9, 4.8; N, 21.8, 22.0. Calc. for $C_{10}H_{12}O_4N_4$: C, 47.6; H, 4.8; N, 22.2%.) No other pure products could be isolated from the oxidation solution.

Ozonolysis. Ozonized O_2 was passed for about 7 hr. through a solution of palitantic acid (0.54 g.) in 0.1N-NaOH (20 ml.) and water (40 ml.). The mixture was now heated to about 60° and a current of air was bubbled through it and then through Brady's reagent. The resulting yellow precipitate proved to be n-butyraldehyde 2:4-dinitrophenylhydrazone. 0.42 g. of impure palitantic acid was recovered from the ozonized solution.

Oxidation of the lactonic acid $C_{13}H_{22}O_4$ from tetrahydropalitantin with nitric acid. Formation of (+)-n-heptylsuccinic acid

The syrupy lactonic acid from tetrahydropalitantin (1.1 g.; Birkinshaw & Raistrick, 1936, p. 807) was heated on the water bath with a mixture of conc. HNO_3 (13 ml.) and water (9 ml.). The reaction was vigorous at first, but gradually slackened and became negligible after 3 hr. The mixture was evaporated on the water bath in an open dish to a syrup. A smell of fatty acids, butyric, hexanoic, etc., was observed. On cooling, a mass of crystals mixed with oil was obtained, wt. 0.90 g. This mass was treated with a little water, well stirred and filtered. The filtrate, which consisted

of an aqueous solution with drops of oil suspended in it, contained much oxalic acid.

The solid (wt. 0.44 g.; m.p. 39–42°) was shown by titration to contain a little unchanged lactic acid. This was removed by repeated solution of the crystals in the titratable amount of *N*-NaOH followed by acidification with HCl. The final product, having a titration equivalent of 111, was recrystallized from ether (1 vol.) followed by the addition of light petroleum (b.p. 40–60°; 10 vol.). The oxidation product was thus obtained as colourless slender rods, m.p. 82–84°, not depressed on admixture with synthetic (±)-*n*-heptylsuccinic acid, m.p. 86° (see next section). (Found: C, 61.1, 61.1; H, 9.0, 9.3; equivalent by titration 108.7. Calc. for $C_{11}H_{20}O_4$: C, 61.1; H, 9.3%; equivalent, titrating as a dicarboxylic acid, 108.1.) $[\alpha]_{D}^{20} = +33^\circ$ (c, 0.97 in ethanol).

Optically active (+)-n-heptylsuccinic acid dihydrazide. The oxidation acid (0.2 g.) was esterified with diazomethane and treated with a solution of hydrazine hydrate (50%; 0.5 ml.) in ethanol (1 ml.). The crystals which appeared overnight were recrystallized from ethanol-ether and were obtained as colourless needles, m.p. 166°. (Found by I.C.I. Butterwick Research Laboratories: C, 53.8, 54.0; H, 9.9, 9.8; N, 22.3, 22.5. $C_{11}H_{21}O_2N_4$ requires C, 54.1; H, 9.9; N, 22.9%.)

Synthesis of (±)-*n*-heptylsuccinic acid

Ethyl 2-bromononanoate (α -bromopelargonate) was condensed with ethyl sodiomalonate, and the resulting triester was hydrolysed and heated to eliminate one carboxyl group.

Preparation of ethyl 2-bromononanoate (Auwers & Bernhardt, 1891). Bromine (10 ml.) was added drop by drop to a mixture of nonanoic acid (15.8 g.) and red P (1.04 g.). The mixture was gradually warmed to 90–100° and more Br (2 ml.) was added. When the evolution of HBr had practically ceased (4–5 hr.) the mixture was cooled and ethanol (30 ml.) was added with cooling. Next day the ethyl α -bromopelargonate was extracted with ether and the ethereal solution was washed with water and aqueous Na_2CO_3 , dried over Na_2SO_4 and distilled *in vacuo*. The ester (22.45 g.) was collected at 135–142°/15 mm.

Condensation of ethyl 2-bromononanoate with malonic ester and hydrolysis of the resulting triester. Na (1.96 g.) was dissolved in ethanol (27 ml.) and malonic ester (13.6 g. = 11.8 ml.) was added. The mixture was warmed slightly and ethyl 2-bromononanoate (22.4 g.) was added, and the whole was refluxed for 2 hr. The resulting ester was extracted in the usual manner and distilled *in vacuo*. Yield 21.7 g.; b.p. 153°/1 mm.

The ester (2 g.) was heated with KOH (5 g.) and water (3 ml.) in a nickel crucible with constant stirring, the temperature of the metal bath being raised to 300° as rapidly as frothing would permit. The melt was cooled, dissolved in water and acidified with HCl. Colourless plates (1.27 g.) separated, m.p. 126–128°.

Decarboxylation of the tricarboxylic acid. The above acid, m.p. 126–128°, was heated in an oil bath when decarboxylation occurred at the melting point (bath temp. 135–140°). After about 10 min. effervescence had practically ceased. The product which crystallized on cooling was neutralized with aqueous *N*-NaOH, filtered and acidified with HCl. The resulting emulsion soon crystallized. The crystals were collected, dried and recrystallized from ether-light petroleum.

(±)-*n*-Heptylsuccinic acid was thus obtained as colourless irregular prisms, m.p. 86°; equivalent by titration 110. (Found by I.C.I. Butterwick Research Laboratories: C, 61.0, 61.0; H, 9.0, 9.2. $C_{11}H_{20}O_4$ requires C, 61.1; H, 9.3%; equivalent 113.)

(±)-*n*-Heptylsuccinic acid dihydrazide. This dihydrazide was prepared in the same way as was the dihydrazide of the oxidation product (+)-*n*-heptylsuccinic acid (see above). It formed colourless needles, m.p. 156°. Although there was a difference of 10° in melting point between the synthetic (±) and the natural dihydrazides, there was no depression in melting point on mixing them.

Reactions with periodate

Action of periodate on tetrahydropalitanin

Oxygen utilization. Tetrahydropalitanin (0.0493 g.) dissolved in water (100 ml.) by warming and subsequent cooling was treated with KIO_4 (50 ml. of 0.0125*M*) and left overnight. Excess H_2SO_4 and KI were then added and the I_2 liberated was titrated with 0.05*N*- $Na_2S_2O_3$. The I_2 deficit as compared with a blank experiment was 6.24 ml. of 0.1*N*, equivalent to a disappearance of four atoms I or utilization of two atoms O/mol. of tetrahydropalitanin.

Acidic groups formed. The experiment was repeated with 0.1616 g. of tetrahydropalitanin using the theoretical amount (100 ml.) of KIO_4 (0.0125*M*), in order to determine the acid and carbonyl groups present after the reaction. After standing overnight, the solution required 11.54 ml. of 0.1*N*-NaOH for neutralization to phenolphthalein. The theoretical figure for 2 equivalents of acid produced in the reaction would be 12.50 ml. The actual figure obtained was always somewhat lower, doubtless owing to lactone formation.

Aldehyde titration

By Doeuvre's method (Doeuvre, 1927). To the neutralized solution was added mercury potassium iodide (3 g.) in a little water with just sufficient KI to obtain complete solution, followed by NaOH (40 ml. of 0.1*N*). A yellow precipitate was formed in the solution. After 3 hr., titration with 0.1*N*-acetic acid containing equimolar sodium acetate showed the disappearance of 16.05 ml. of alkali. The aldehyde present is equivalent to 5.35 ml. (= $\frac{1}{2}$ of 16.05), whereas the theoretical value for 1 CHO = 6.25 ml.

By bisulphite titration (Malaprade (1934) method). The periodate oxidation was repeated (0.1616 g. of tetrahydropalitanin; 100 ml. of 0.0125*M*- KIO_4). 11.20 ml. of 0.1*N*-NaOH were required for neutralization of the acid produced in the reaction. Bisulphite (30 ml. about 0.2*M*) was then added, the solution was left for 1 hr. with exclusion of air, and was then titrated. It required 15.90 ml. of 0.05*M*-iodate. The bisulphite taken was equivalent (by titration) to 45.04 ml. of 0.05*M*-iodate, the difference being 29.14 ml. Of this 25.00 ml. would be derived from the periodate. The iodate corresponding with aldehyde is, therefore, 4.14 ml. whereas 1 CHO group would require 4.17 ml.

By hydroxylamine titration (Schultes, 1934). The same method of oxidation was followed and the same quantities used, but after removal of the iodate with H_2SO_4 and KI and titration of the liberated I_2 with thiosulphate, the solution was neutralized to bromophenol blue, and a solution of about 1 g. of NH_2OH , HCl neutralized to the same end point was added. The mixture immediately became acid and was

titrated from time to time with 0.1N-NaOH until permanent neutrality (to the same end point) was reached. The alkali used was 6.7 ml., whereas 1 CHO group requires 6.25 ml.

There is ample evidence to show the production (or persistence) of 1 CHO group on periodate oxidation.

Production of lactone group. The production of a lactone group is shown more clearly in the following experiment. Tetrahydropalitantin (0.25 g.) in 25 ml. of methanol was treated with KIO_4 (0.5 g.) in water (125 ml.). A turbidity appeared in 5–10 min. which gradually increased. After standing overnight the mixture was titrated to phenolphthalein. It used 16.8 ml. of 0.1N-NaOH to the first pink flush, but required 5–6 hr. to reach a permanent end point, when 20.5 ml. of NaOH had been utilized, the theoretical figure for 2 equiv. of acid produced being 19.4 ml. After standing overnight a further 0.30 ml. of 0.1N alkali was required.

Characterization of the periodate oxidation product of tetrahydropalitantin. The product obtained by the oxidation of tetrahydropalitantin (0.25 g.) with KIO_4 (0.5 g.) was extracted with ether, suspended in water (100 ml.) and treated with Doeuvre's reagent (0.45 g. of HgI_2 , 2 g. of KI in a little water). Then N-NaOH (10 ml.) was introduced, the mixture was well shaken and left overnight. The precipitated Hg was filtered off and the solution titrated. It required 6.40 ml. of N-HCl; the acid produced in the reaction is therefore 3.60 ml. (1 CHO group requires 3.88 ml.). The solution was now acidified with HCl and extracted with ether. The ether extract was esterified by means of diazomethane. The resulting oil (which contained a little Hg salt) was treated with ethanol (2 ml.) and hydrazine hydrate (50%, 1 ml.). The solid mass of crystals obtained (0.17 g.) was drained at the pump, and recrystallized from ethanol giving 0.12 g. of pure product of m.p. 190°. This gave no depression when mixed with the product $\text{C}_{13}\text{H}_{22}\text{O}_3\text{N}_4$ obtained previously by the oxidation of tetrahydropalitantin with Ag_2O (Birkinshaw & Raistrick, 1936, p. 807).

In a larger scale experiment tetrahydropalitantin (1.613 g.) was oxidized by KIO_4 (2.875 g.) and the aldehydic product further oxidized with Doeuvre's reagent as above. In this case the mercury precipitated was weighed and acid produced in the Doeuvre reaction was determined (Hg 1.23 g. 1 CHO requires 1.25 g.; acid formed 17.9 ml. 1 CHO requires 18.75 ml.).

The solution was acidified to Congo red with H_2SO_4 and extracted with CHCl_3 (4 × 2 l.). After removal of solvent *in vacuo* the residue (1.22 g.) was taken up in ether, filtered from a small amount of HgI_2 , taken up by the CHCl_3 and esterified by means of diazomethane. The product, distilled *in vacuo* afforded 0.84 g. of distillate b.p. 175°/3 mm. with methoxyl content 12.6%. $\text{C}_{14}\text{H}_{22}\text{O}_4$ requires (for 1 OMe) 12.1%. This represents the monomethyl ester of the lactone acid.

The distillate (0.79 g.) was treated with phenylhydrazine (0.5 g.) in ethanol (2 ml.) and heated under reflux for 2 hr. The product was treated with ether and the solvent allowed to evaporate slowly. A small amount of crystals was obtained, m.p. 192°, raised on recrystallization to 206°. (Found: C, 67.8; H, 8.2; N, 12.8. $\text{C}_{23}\text{H}_{34}\text{O}_3\text{N}_4$ requires C, 68.1; H, 8.2; N, 12.7%. The product is evidently the bisphenylhydrazide of the lactonic acid $\text{C}_{13}\text{H}_{22}\text{O}_4$.)

Action of periodate on palitantin

Palitantin (0.0594 g.) in ethanol (25 ml.) was treated with KIO_4 (0.0125M; 100 ml.) and H_2SO_4 (2N; 10 ml.). After 20 hr. at room temperature excess KI was added and the I_2 was titrated with 0.1N- $\text{Na}_2\text{S}_2\text{O}_3$. The I_2 deficit as compared with a blank was 13.03 ml. 0.1N corresponding with 5.74 atoms I/mol. of palitantin. Part of the I_2 uptake is due to I_2 substitution, which is a characteristic property of palitantin and presumably of the fission product.

When the theoretical amount of KIO_4 for an uptake of 4 I atoms (=2O) was used and there was no subsequent liberation of free I_2 the results were similar to those obtained with tetrahydropalitantin.

Evidence of lactone formation. Palitantin (1 g.) in methanol (100 ml.) treated with KIO_4 (2 g.) in water (600 ml.) remained clear. After 24 hr. it required 6.4 ml. of N-NaOH to the first pink flush of phenolphthalein gradually increasing to 7.95 ml. over 3 hr. The theoretical value for 2 equivalents of acid produced is 7.87 ml. This is again evidence for one product being a lactone.

Aldehyde production. The reaction mixture was acidified with 10 ml. of 2N- H_2SO_4 and extracted with ether. The oily residue obtained on evaporation of the ether was suspended in water (about 200 ml.) and neutralized to bromophenol blue. After addition of a solution of $\text{NH}_4\text{OH} \cdot \text{HCl}$ (2 g.) at the same pH the solution was titrated at intervals until a permanent end point was reached, matching to the same arbitrary standard. The NaOH required was 3.4 ml., the theoretical amount for 1 aldehyde group being 3.88 ml. Practically all the emulsion dissolved leaving only a trace of turbidity.

Oxidation of aldehyde group and characterization of product. The product from the KIO_4 (2 g.) oxidation of palitantin (1 g.) was extracted with ether after acidifying. The residual syrup after removal of ether was oxidized with Doeuvre's reagent. Barium acetate was added and the oxidation product was precipitated as the barium salt by addition of 4 vol. of ethanol. The Ba precipitate was collected, suspended in water, acidified with HCl and the acid product extracted with ether. The product was then esterified by means of diazomethane and treated with ethanol (4 ml.) and hydrazine hydrate (50%; 2 ml.). The crystals obtained (0.47 g.; m.p. 202–204°) showed no depression when mixed with the dihydrazide $\text{C}_{13}\text{H}_{24}\text{O}_3\text{N}_4$ obtained by oxidation of palitantin with Ag_2O as previously described (Birkinshaw & Raistrick, 1936).

Action of periodate on tetrahydropalitantic acid

Utilization of oxygen. Tetrahydropalitantic acid (0.0857 g.) suspended in water (25 ml.) was neutralized to phenolphthalein to bring it into solution and treated with KIO_4 (0.0125M; 50 ml.) and left overnight. Excess of H_2SO_4 and KI were then added and the liberated I_2 was titrated with $\text{Na}_2\text{S}_2\text{O}_3$. The deficiency of I_2 was 6.44 ml. of 0.1N as compared with a blank, corresponding with a utilization of 2.06 atoms of I ($\equiv 1.03$ atoms of O).

Determination of acid and carbonyl groups. Tetrahydropalitantic acid (0.1713 g.) was dissolved in the theoretical amount of NaOH (0.1N; 6.25 ml.) and KIO_4 (0.0125M; 50 ml.; i.e. the theoretical amount) was added. After 2 days it required 0.11 ml. of 0.1N-NaOH to neutralize to phenolphthalein, i.e. no acid is formed in the reaction. To the titrated solution were added H_2SO_4 (0.1N; 50 ml.) and

excess KI. Titration with $\text{Na}_2\text{S}_2\text{O}_3$ showed a deficiency of 12.48 ml. of 0.1 N- I_2 corresponding with 2 atoms of I (theoretical 12.50 ml.). The solution was then treated with bromophenol blue and brought to an arbitrary end point (faint purple colour) with NaOH. NH_2OH , HCl (1 g. in 10 ml. of water) neutralized to the same end point was added. After 2 hr. the alkali required to establish the same end point was 11.5 ml. of 0.1 N-NaOH. The theoretical value for two aldehyde groups is 12.5 ml. Thus two carbonyl groups are produced in the reaction, provided no such groups titratable with NH_2OH are present in the original tetrahydropalitanic acid. The absence of such groups was shown by the addition of the NH_2OH reagent to another sample of the acid. No acidity was produced.

Isolation of frequentin

The crude crystalline product (11.75 g.) obtained by CHCl_3 extraction of the culture solution from two batches of *P. palitans* type strain (Birkinshaw & Raistrick, 1936) was dissolved in CHCl_3 (500 ml.) and 100 ml. of water were added. N-NaOH was added dropwise with vigorous shaking until the aqueous layer was just alkaline to phenolphthalein. This required 11.5 ml. of N-NaOH. The aqueous layer was separated and acidified with N-HCl (12 ml.). The oil precipitated solidified on chilling and was crude frequentin, wt. 2.52 g. A small amount was recovered from the mother liquor.

Frequentin was difficult to recrystallize as it decomposed on heating for any length of time. It was purified for analysis by recrystallizing in small amounts from boiling water, with addition of decolorizing charcoal. Frequentin crystallized in colourless needles, m.p. 134.5° (decomp.), $[\alpha]_{\text{D}}^{20} + 82^\circ$ in CHCl_3 . On microtitration 10.260 mg. required 2.80 ml. N/70-NaOH, equiv. 257. (Found: C, 66.0, 66.0, 66.5, 66.4; H, 7.7, 7.8, 8.2, 8.2. Calc. for $\text{C}_{14}\text{H}_{20}\text{O}_4$: C, 66.6; H, 8.0%, equiv. 252.) Frequentin gives a reddish

purple colour with FeCl_3 in aqueous or alcoholic solution and yields a precipitate with Brady's reagent.

A comparison of our product obtained from *P. palitans* Westling with frequentin obtained from *P. frequentans* (Curtis *et al.* 1951), kindly carried out by Mr P. J. Curtis, indicated that the two products were identical since there was no depression in melting point on mixing.

SUMMARY

1. A number of derivatives and breakdown products of palitantin have been prepared and are described.

2. Palitantin, contrary to the previous statement (Birkinshaw & Raistrick, 1936), has a slight but definite optical activity.

3. The oxidation of palitantin and its derivatives with periodate has been studied in a quantitative manner.

4. Tetrahydropalitanin probably consists of a 6-carbon ring with a *n*-heptyl and a formyl group as substituents and with hydroxyl groups situated α and β to the formyl group. In palitantin one double bond is in the C_7 chain in the γ position to the terminal methyl group. The function of the fourth oxygen atom and position of the second double bond (if present) are still obscure.

5. The acidic substance which accompanies palitantin as a metabolic product of *Penicillium palitans* is identical with frequentin isolated from *Penicillium frequentans* by Curtis *et al.* (1951).

I have to thank the Butterwick Research Laboratories of I.C.I. Ltd. for the elementary analyses of two products. Other micro-analyses are by Weiler and Strauss, Oxford.

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A Structural Relationship between Frequentin and Palitantin

By P. J. CURTIS AND L. A. DUNCANSON

Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn, Herts

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Birkinshaw & Raistrick (1936), in describing the isolation of palitantin ($\text{C}_{14}\text{H}_{22}\text{O}_4$) from *Penicillium palitans*, mention the occurrence of a dextrorotatory acid in concentrates from one strain. Curtis, Hemming & Smith (1951) isolated a substance named frequentin ($\text{C}_{14}\text{H}_{20}\text{O}_4$) together with palitantin from certain atypical strains of *Penicillium frequentans*. Subsequently it was pointed out to us

by Dr J. H. Birkinshaw (cf. Birkinshaw, 1952, preceding paper) that the dextro-rotatory acid was very probably identical with frequentin, and we have established this by a mixed melting point determination and by comparing the infrared spectra of the two substances.

In common with palitantin, frequentin has an aldehyde group, an ethylenic double bond or bonds

and three active hydrogens, but is distinguished from it by the presence of an enolic hydroxyl. This paper presents evidence that the carbon skeletons and the positions of the oxygen atoms are identical.

Reduction of frequentin and palitantin

Frequentin was reduced by sodium amalgam in acid solution giving a tetrahydro-derivative ($C_{14}H_{22}O_4$), m.p. 170–171°, which no longer contained a formyl group, or an enolic hydroxyl. Palitantin was also reduced by sodium amalgam giving dihydropalitantin ($C_{14}H_{22}O_4$), m.p. 169°. No depression of melting point occurred on mixing these reduction products.

Infrared spectra of palitantin, frequentin and dihydropalitantin

The spectra of dihydropalitantin and the tetrahydro-derivative of frequentin are the same in all respects. Hence these two substances are identical, and it follows that palitantin and frequentin have the same carbon skeleton and that their oxygen atoms are in corresponding positions.

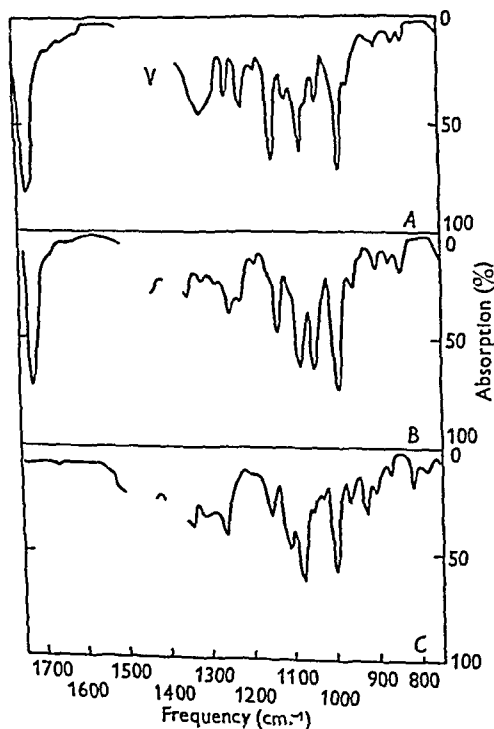


Fig. 1. Infrared absorption spectra. A, frequentin; B, palitantin; C, dihydropalitantin.

The absorption curves, from 750 to 1750 cm^{-1} of frequentin, palitantin and dihydropalitantin are shown in Fig. 1. Bands due to $C=O$ stretching modes occur in the 6μ . (1600 cm^{-1}) region; thus as dihydropalitantin has no band in this region,

carbonyl groups are absent. Frequentin has an absorption band at 1732 cm^{-1} with a slight shoulder at about 1720 cm^{-1} , whilst palitantin has a band at 1718 cm^{-1} . This confirms that there are at least two carbonyl groups in frequentin, and at least one in palitantin. The fact that these frequencies are above 1710 cm^{-1} , together with chemical evidence, indicates that unless the carbonyl groups form part of a strained ring, they are not conjugated with other double bonds.

There are quite strong similarities between the spectra of the three compounds, some bands being traceable through them all with only slight modifications of their frequencies. As would be expected, in the region 1000–1200 cm^{-1} marked differences appear. In this region absorptions due to vibrational modes involving deformation of alcoholic $O-H$ groups occur, and whilst three fairly strong bands are present in all three spectra (i.e. near 1000 cm^{-1} , near 1080 cm^{-1} and near 1140 cm^{-1}), variations in this frequency range are apparent.

The 3μ . (3300 cm^{-1}) regions in which $C-H$ and $O-H$ stretching frequencies occur are not shown in the diagram, but bands due to alcoholic hydroxyl groups occur at the following frequencies: frequentin, 3420 cm^{-1} ; palitantin, 3370 cm^{-1} ; dihydropalitantin, 3350 cm^{-1} .

EXPERIMENTAL

Impure samples of frequentin and palitantin were freed from each other by virtue of the fact that frequentin alone is extracted from $CHCl_3$ solution by Na_2CO_3 solution.

Reduction of frequentin. Frequentin (250 mg.) was dissolved in hot water (500 ml.), and, after cooling, 25 g. KH_2PO_4 were added, then 100 g. of 2.5% Na amalgam in about thirty portions during 2 hr. The mixture was extracted three times with 200 ml. $CHCl_3$, and the extracts after shaking with $N-Na_2CO_3$ solution saturated with NaCl were combined and concentrated giving colourless plates (35 mg.) m.p. 164–165°. One recrystallization raised the melting point to 170–171°, but no change was observed on further recrystallization. (Found C, 65.3; H, 9.4; mol. wt. 283. $C_{14}H_{22}O_4$ requires C, 65.6; H, 9.4%; mol. wt., 256.) The compound did not reduce ammoniacal Ag_2O nor did it give a colour with $FeCl_3$. It decolorized a solution of Br_2 in glacial acetic acid.

Reduction of palitantin. Palitantin (500 mg.) was dissolved in ethanol (50 ml.) and 2.5% Na amalgam (100 g.) with a slight excess of $N-H_2SO_4$ was added in ten portions during 6 hr. After standing 1 hr. the mixture was boiled and then treated similarly to that from frequentin. Yield 154 mg., m.p. 162–164°. (Found C, 65.7; H, 9.4. $C_{14}H_{22}O_4$ requires C, 65.6; H, 9.4%.) Recrystallization from water or $CHCl_3$ was unsatisfactory. A sample (m.p. 165°) on admixture with a sample of the tetrahydro derivative of frequentin (m.p. 166–167°), melted at 167°. Purification by fractional extraction between $CHCl_3$ and water gave a solid, m.p. 169°.

Oxidation of dihydropalitantin. The reduction product was found to oxidize slowly in air at room temperature, and after

several weeks a smell like that of butyric acid was noticed. The samples prepared for analysis in the above work were therefore kept *in vacuo* or in N_2 .

Infrared spectra

Measurements were made with a modified Hilger D. 209 spectrometer, fitted with a rock-salt prism and working as a single beam instrument. Absorption due to atmospheric water vapour was minimized by enclosing the instrument in an air-tight Perspex box through which dry air was circulated. The samples were ground to fine powders which were moistened with Nujol, the resulting mulls being held as thin films between rock-salt plates for examination.

The intensity of the radiation incident on the samples was determined in the usual way by replacing the samples with two rock-salt plates of the same thickness as those used for supporting the mulls. Absorption curves were then drawn (see Fig. 1), the gaps in which are due to regions of Nujol absorption. For calibration of the spectrometer, hydro-

carbon bands of known frequency were used in conjunction with bands due to CO_2 and a small amount of water vapour present in the instrument. Frequencies below 1750 cm^{-1} are accurate to $\pm 3\text{ cm}^{-1}$, and in the 3300 cm^{-1} region to $\pm 10\text{ cm}^{-1}$.

SUMMARY

The mould metabolites frequentin ($C_{14}H_{20}O_4$) and palitantin ($C_{14}H_{22}O_4$) obtained from *Penicillium palitans* and some atypical strains of *Penicillium frequentans* both give dihydropalitantin ($C_{14}H_{24}O_4$) on reduction with sodium amalgam. Hence the carbon skeletons and positions of the oxygen atoms in the two metabolites are identical.

We are indebted to Mr J. F. Grove for valuable advice, to Dr J. H. Birkinshaw for a sample of the dextrorotatory acid, and to the Directors of I.C.I. Ltd. (Plastics Division) and Mr H. A. Willis for spectrographic facilities.

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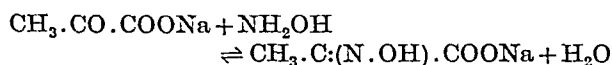
Oxidation of Pyruvic Acid Oxime by Soil Organisms

By J. H. QUASTEL, P. G. SCHOLEFIELD AND J. W. STEVENSON

Research Institute, Montreal General Hospital, Montreal, and Department of Bacteriology and Immunology, McGill University, Montreal

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The possibility that hydroxylamine might be an intermediate in the oxidation of ammonia to nitrite by *Nitrosomonas* has often been discussed. There has been, however, no direct evidence to confirm this view and, indeed, hydroxylamine is well known to be toxic in low concentrations to these and many other organisms. Recently, Lees & Quastel (1946), working on the hypothesis that the hydroxylamine formed might be bound as an oxime, reported that the perfusion of an equimolar mixture of sodium pyruvate and hydroxylamine over a soil enriched with nitrifying organisms through previous perfusions of ammonium chloride, resulted in the formation of nitrate. They suggested that the equilibrium of the reaction between sodium pyruvate and hydroxylamine might be such that only a very low concentration of free hydroxylamine was present at any instant. This



concentration might be sufficiently low to allow oxidation of the free hydroxylamine and yet

eliminate its toxic effects. Subsequently, Quastel & Scholefield (1949) were able to show that neither *Nitrosomonas* nor *Nitrobacter* was responsible for oxidation of pyruvic acid oxime (hereafter called pyruvic-oxime) and predicted the existence of a separate enzymic mechanism. The responsible organisms have been isolated, and a preliminary account of them has been reported (Quastel, Scholefield & Stevenson, 1950). The present communication gives a more detailed account of these organisms and of the metabolism of pyruvic-oxime by one of them. Jensen (1951) has since reported the isolation of other organisms capable of producing nitrite from pyruvic-oxime.

METHODS

Preparation of materials. The preparation of the soil used in these experiments, the general methods and the estimations used in soil perfusion have already been fully described (Lees & Quastel, 1946; Quastel & Scholefield, 1949, 1951). A local soil was used throughout the present experimental work.

D-Arabinose-oxime was kindly supplied to us by Dr S. B. Baker, of this Institute, and formaldoxime, salicyl-

aldoxime, furil dioxime and benzoin oxime were Analar reagents. The oximes of acetone and acetoacetic ester were not isolated, a mixture of 1.1 moles of the ketone with 1.0 mole of hydroxylamine being used for the perfusion experiments.

The oximes of the sodium salts of pyruvic, oxaloacetic, α -ketoglutaric and phenylpyruvic acids were prepared as follows: hydroxylamine hydrochloride (1.0 mole) and the free acid (1.1 moles) were mixed together and a little NaHCO_3 was added. The mixture was kept as a thick paste

and arabinose oxime, do not yield appreciable quantities of nitrite in a period of 6 weeks. Chlorate is added in all cases to inhibit the further oxidation of nitrite (Lees & Quastel, 1945). Representative results are given in Table 1. It will be noted from this table that the oximes of oxaloacetic, phenylpyruvic and α -ketoglutaric acids do not inhibit markedly (if at all) the oxidation of pyruvic-oxime in soil. Similarly, D-arabinose oxime, whilst itself

Table 1. Nitrite accumulation after perfusion of oximes through a soil

(μg . Nitrite N accumulating per ml. perfusate after perfusion of various oximes (0.01 M) through soil in the presence of 0.001 M-sodium chlorate. 0.01 M-Oxime = 140 μg ./ml.)

Time in hr.	Exp. 1 Exp. 2 Exp. 3	42 43	70 66	91 91	114 115	139 138	300
0.01 M-Pyruvic-oxime	Exp. 1 Exp. 2 Exp. 3	48 6	113 16	113 105	110 107	114 110	109
0.01 M-Arabinose oxime	Exp. 1	4	9	9	11	10	
0.01 M-Arabinose oxime + 0.01 M-pyruvic-oxime	Exp. 1	52	112	109	111	111	
0.01 M- α -Ketoglutaric acid oxime	Exp. 2	11	29	89	104	109	
0.01 M- α -Ketoglutaric acid oxime + 0.01 M-pyruvic-oxime	Exp. 2	3	36	103	189	200	
0.01 M-Oxaloacetic acid oxime	Exp. 3						98
0.01 M-Oxaloacetic acid oxime + 0.01 M-pyruvic-oxime	Exp. 3						203
0.01 M-Phenylpyruvic acid oxime	Exp. 1	1	22	40	58	62	
0.01 M-Phenylpyruvic acid oxime + 0.01 M-pyruvic-oxime	Exp. 1	57	132	150	154	153	
0.01 M-Salicylaldoxime	Exp. 3						3
0.01 M-Furil dioxime	Exp. 3						2

by the addition of small quantities of water as needed. Further NaHCO_3 was added until no more CO_2 was evolved and then sufficient water was added to bring the paste into solution at the boiling point in the presence of a slight excess of the alkali. On cooling, the solution deposited crystals of the sodium salts of the oximes of these α -ketoacids and it was in this form that they were used. The oxime of sodium pyruvate was generally recrystallized from boiling water to remove the brown colour normally associated with the pyruvic acid used. The samples of pyruvic-oxime used in this work contained no free pyruvate as estimated by yeast carboxylase and no free hydroxylamine as estimated by MnO_2 (Mann & Quastel, 1946). α -Ketobutyric acid oxime was prepared by Dr L. Martin of this Institute.

RESULTS

Perfusion of oximes through soil

The first oxime which was found to undergo metabolism in soil was pyruvic-oxime (Lees & Quastel, 1946; Quastel & Scholefield, 1949). It was later shown that oxaloacetic acid oxime suffers a similar breakdown (Quastel *et al.* 1950). To these must now be added phenylpyruvic acid oxime and α -ketoglutaric acid oxime, whilst salicylaldoxime, furil dioxime

yielding little nitrite, in no way impedes the oxidation of pyruvic-oxime. Re-perfusion of arabinose oxime yielded no nitrite in 4 days. The stability of other sugar oximes in soil is now under consideration. Whilst only a dozen oximes have been studied to date, it seems likely that only those of the α -ketoacids are able to undergo nitrification in soil. A preliminary experiment has also shown that the hydrazones, unlike the oximes of these acids, are not nitrified.

The oximes of acetone, formaldehyde, acetoacetic ester and benzoin are relatively inactive as nitrite formers in soil and do not inhibit the oxidation of pyruvic-oxime. It has also been found that disodium succinohydroxamate (prepared in this Institute by Dr L. Martin) does not inhibit this oxidation.

Certain antibiotics have been tested as possible inhibitors of pyruvic-oxime oxidation in soil. It has been found that penicillin and streptomycin are relatively inert. Thus, at a concentration of 0.01 % they produce only half a day's lag period with pyruvic-oxime as substrate. Chloromycetin produces a lag period varying from half a day at a concentration of 0.001 % to 6 days at 0.05 %.

The values for the lag periods are obtained by finding the difference between the times required to reach the asymptotic nitrite value in the presence and absence of the inhibitor. It is of interest to note that, at a concentration of 0.025 %, chloromycetin entirely prevents oxidation of sodium nitrite in soil for a period of more than 10 days (Quastel & Scholefield, 1951).

Nitrourea at a concentration of 0.0033M completely inhibits nitrite oxidation for 16 days whilst having no effect on pyruvic-oxime oxidation (Quastel & Scholefield, 1951). Similarly, *p*-aminosalicylic acid produces a 1.5-day lag period in the oxidation of pyruvic-oxime in soil at a concentration of 0.0033M, but will produce a similar effect on the oxidation of ammonium ions at a concentration of 0.001M (Quastel & Scholefield, 1951).

At a concentration of 0.005 % the only sulphadiazine drug to have any effect on pyruvic-oxime oxidation is sulphadiazine, six others having relatively little effect.

Manometric experiments on washed suspensions of *Achromobacter*

As will be seen from the Addendum to this paper, four organisms have been isolated, three from soil and one from faeces, which can bring about the oxidation of pyruvic-oxime to nitrite. Manometric experiments have been carried out using suspensions of these organisms.

Pyruvic-oxime oxidase

The fact that the oxidation of pyruvic-oxime, which takes place in the presence of these organisms, is controlled by a thermolabile enzyme was established by showing that immersion of the organisms in a boiling-water bath for 1 min. completely eliminated their respiratory activity in the presence of pyruvic-oxime. Furthermore, the presence of M/300 sodium cyanide inhibited the oxygen uptake by over 70 % and M/300 sodium azide by over 90 %. The enzyme controlling the oxidation was termed pyruvic-oxime oxidase (Quastel *et al.* 1950).

Whilst it was quite definite that the three soil organisms and the one isolated from faeces could all metabolize pyruvic-oxime and produce nitrite from this source, their relative activities differed considerably. Suspensions of each organism were therefore prepared and the rates of oxygen uptake in air were measured using potassium hydroxide in the centre wells of the Warburg vessels. In each case the turbidities were made the same, as judged with the Fisher colorimeter using Filter B525, and in each case the endogenous metabolic rate was also estimated. Fig. 1 shows that both *Achromobacter* sp. (1) and the faecal organism actively metabolize pyruvic-oxime. The other species of *Achromobacter* and the species of *Corynebacterium*, on the other hand,

showed only a low rate of oxidation of pyruvic-oxime under these conditions. In all the experimental work discussed in this paper, therefore, the *Achromobacter* sp. (1) has been used. The organisms were always washed twice with distilled water and suspended in distilled water. The suspension contained the growth from one 3-day-old plate per ml. Approximately 5 mg. dry weight washed organisms was used per vessel in all experiments.

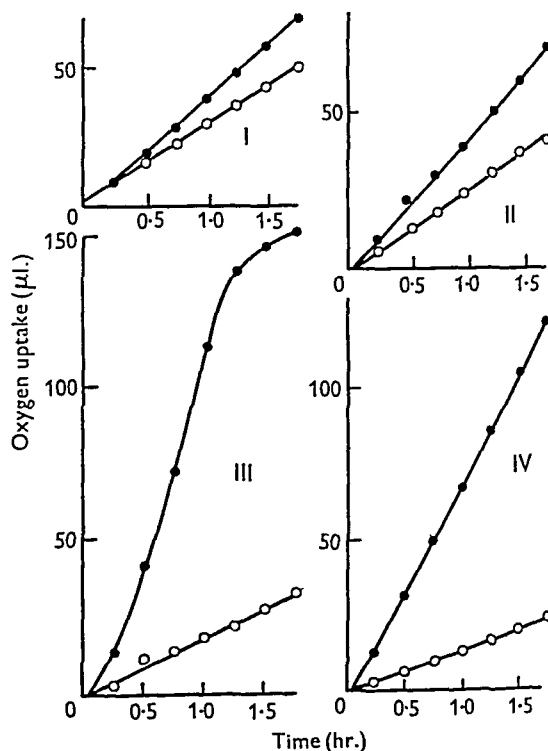


Fig. 1. Comparison of the abilities of four organisms to oxidize pyruvic-oxime. Contents of flasks: 1.0 ml. washed suspension of organisms (5 mg. dry wt.), 1.0 ml. 0.1M-phosphate buffer, pH 7.2; 0.2 ml. 0.02M-pyruvic-oxime; water to total vol. 3.2 ml. Air 37°. KOH in centre wells. (I) *Corynebacterium* sp. from soil; (II) *Achromobacter* sp. (2) from soil; (III) *Achromobacter* sp. (1) from soil; (IV) *Achromobacter* sp. (3) from faeces; O, no substrate added; ●, 0.2 ml. 0.02M-pyruvic-oxime present.

In view of our previous studies concerning the effects of hydrogen-ion concentration on the production of nitrite in soils (Quastel & Scholefield, 1949, 1951) it seemed of interest to investigate the effects of changes of pH on the oxidation of pyruvic-oxime. It will be seen from Fig. 2 that in the range of pH 6–9 there is no appreciable variation in the rate of O_2 consumption, but that a shift of 0.5–1.0 pH unit outside this range causes a very sharp drop in activity. In all our experiments on the production of nitrite from pyruvic-oxime in soil, therefore, pH may be eliminated as a factor in-

fluencing metabolic activity since the buffering power of soil is great enough to maintain conditions well within the region of pH 6-9.

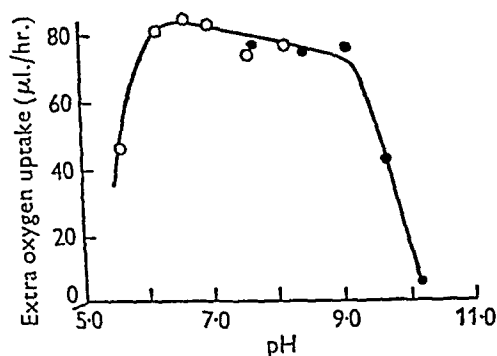


Fig. 2. The variation of activity of pyruvic-oxime oxidation with pH. O, phosphate buffer (0.066M); ●, borate buffer (final concentration of H_3BO_3 and KCl 0.05M; pH adjusted with NaOH). For experiment: 2.0 ml. buffer solution; 1.0 ml. bacterial suspension; 0.2 ml. 0.25M-pyruvic-oxime or 0.2 ml. water tipped.

Oxygen consumption during the oxidative metabolism of pyruvic-oxime

It was of interest to discover the number of molecules of O_2 consumed by the organism during its metabolism of one molecule of pyruvic-oxime and whether the ratio of O_2 consumed to pyruvic-oxime used remained constant during the course of an experiment. It has been found convenient to estimate the amount of nitrite formed, as this is a measure of the metabolism of pyruvic-oxime.

Table 2. Comparison of oxygen uptake and nitrite production during metabolism of pyruvic-oxime

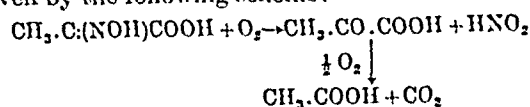
(Details given in text.)

Time (min.)	Extra oxygen uptake (i) (μl.)	Nitrite produced (ii) (1 g.mol. = 22.4 l.) (μl. equivalent)	Ratio: (i)/(ii)
15	4.1	2.24	1.8
30	7.4	4.62	1.6
45	13.6	7.54	1.8
60	21.8	—	—
75	30.0	15.3	1.95
90	35.7	20.2	1.8
105	55.4	31.2	1.9
120	71.0	37.6	1.9
135	79.5	41.5	1.9
		Mean	1.82

The results of a typical experiment are given in Table 2. Several manometer vessels were set up containing 1.0 ml. bacterial suspension of the standard organisms, 1.0 ml. 0.1 M-phosphate buffer, pH 7.2, and 1.0 ml. water; and into each of them was tipped 0.2 ml. 0.2M-pyruvic-oxime. Every 15 min. one vessel was removed and the nitrite content of

the medium was estimated colorimetrically. From the results given in the above table it will be seen that 1.82 molecules of O_2 are consumed per molecule of sodium nitrite produced. The value of this figure indicates that the molecule of pyruvic-oxime is not completely oxidized by these organisms under our experimental conditions. The theoretical value of the ratio in question for complete oxidation to CO_2 , water and nitrite would be 3.5. Furthermore, it is obvious that the ratio of nitrite produced to O_2 consumed is constant within experimental error during the entire experiment.

A likely mode of breakdown of pyruvic-oxime is given by the following scheme:



Such a scheme involves the necessary intermediate formation of pyruvic acid. Experiments were therefore carried out to discover whether the presence of semicarbazide, by its binding of intermediary pyruvic acid, would influence the rate of O_2 uptake of *Achromobacter* in the presence of pyruvic-oxime. The results showed that semicarbazide (0.05M) neither altered the rate of O_2 uptake nor, within experimental error, the total O_2 uptake.

The oxygen uptake due to the addition of other substances

(a) *Nitrogen-containing molecules.* The metabolism of a number of substrates by *Achromobacter* sp. (1) has been studied with the intention of discovering which compounds are possible intermediates in the metabolism of pyruvic-oxime. Thus one pathway of metabolism of pyruvic-oxime might be via alanine by reduction of the oxime to this amino-acid. That this is not the case, however, is shown by the facts: (i) that the metabolism of the oxime in soil does not proceed via ammonia, whereas that of alanine does (Quastel & Scholefield, 1949); (ii) that alanine on incubation with *Achromobacter* sp. (1) produces no nitrite although it is quite readily oxidized. Another possible intermediate is hydroxylamine, but previous evidence has already rendered this possibility unlikely. Again 0.0001M-hydroxylamine does not produce nitrite on perfusion in soils that readily oxidize pyruvic-oxime.

Since it is clear that neither ammonia nor hydroxylamine is likely to be an intermediate in the breakdown of pyruvic-oxime and since the ratio of O_2 uptake to the formation of nitrite is constant from the start of the experiment, it is reasonable to deduce that the nitrite is formed by an initial direct oxidation of the oxime.

(b) *Oxidation of possible intermediates in the metabolism of pyruvic-oxime.* Investigations were made of the rates of O_2 uptake by a washed suspension of

Achromobacter sp. (1), in the presence of a variety of substances which might be possible intermediates in pyruvic-oxime metabolism. Sodium lactate and sodium pyruvate were found to be oxidized by the organism without any initial time lag; the rates of O_2 uptake were not constant but tended to increase

would yield another 1.79×0.75 , or 1.34, atoms of O per molecule of pyruvic-oxime (Table 3). Thus the oxidation of the oxime by these organisms would require $1.50 + 1.34$, or 2.84, atoms of O per molecule and this is in agreement with the observed value of

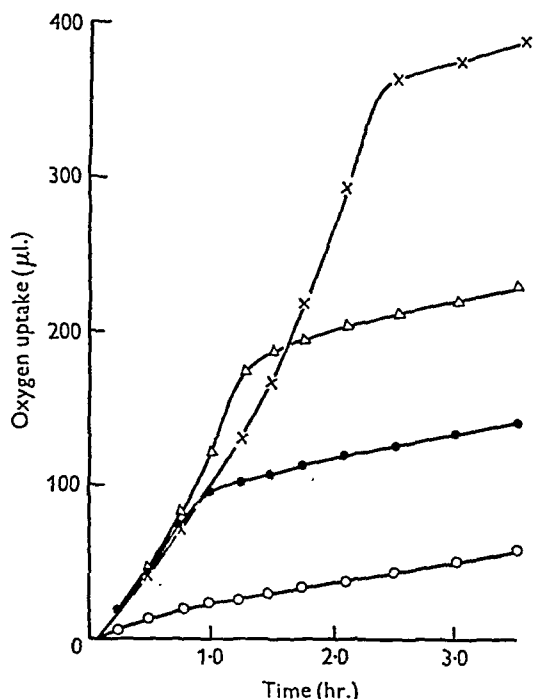


Fig. 3. The effect of pyruvic-oxime concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but with varying amounts of pyruvic-oxime. O, no pyruvic-oxime present; ●, 0.1 ml. 0.02M-pyruvic-oxime; △, 0.2 ml. 0.02M-pyruvic-oxime; ×, 0.4 ml. 0.02M-pyruvic-oxime.

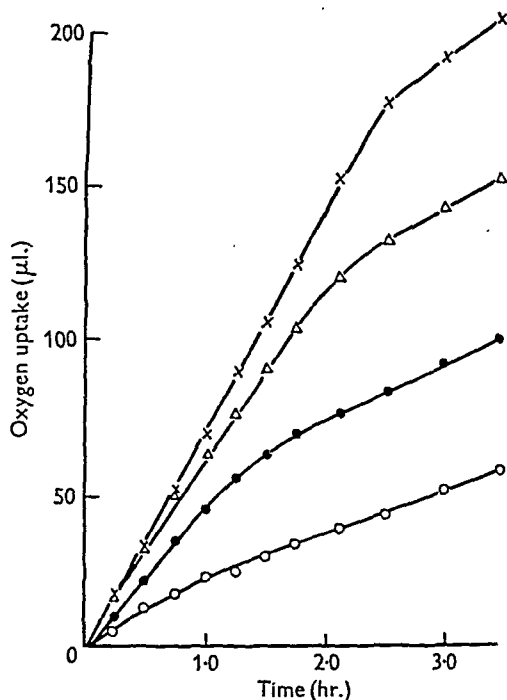


Fig. 4. The effect of sodium pyruvate concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but no pyruvic-oxime present. O, no sodium pyruvate present; ●, 0.1 ml. 0.02M-sodium pyruvate; △, 0.2 ml. 0.02M-sodium pyruvate; ×, 0.4 ml. 0.02M-sodium pyruvate.

with time, a phenomenon also shown by pyruvic-oxime. Sodium propionate was also oxidized, but the onset of oxidation is preceded by an initial time lag. Constant rates of O_2 uptake have been obtained with acetate, but usually even this substrate tends to give an increasing rate of O_2 uptake with time. Typical results for pyruvic-oxime and sodium pyruvate oxidation are given in Figs. 3 and 4. The results shown in Table 3 were obtained with a freshly harvested suspension of organisms that had been subcultured from a stock stored for 6 months at room temperature after lyophilization. Enzymic activity with pyruvic-oxime as substrate remained high but the extent of oxidation was somewhat lowered.

In this experiment the nitrite yield was 75% of the theoretical. It would therefore be expected that 1.50 atoms of O would be taken up per molecule of pyruvic-oxime added, on the assumption that oxidation proceeds initially to pyruvate and nitrite. The further oxidation of the pyruvate produced

2.81. This observation conforms with the view that pyruvate is an essential intermediate in the oxidation of pyruvic-oxime.

Table 3. Atoms of oxygen taken up per molecule of substrate, after lyophilization and storage of *Achromobacter* sp. (1)

Pyruvic-oxime	2.81
Sodium pyruvate	1.79
Sodium acetate	2.24

It will be noted from the results given in Table 3 that the O_2 uptake per molecule of substrate is of the same order with sodium acetate as with sodium pyruvate. This result we consider explicable on the basis that part of the pyruvate (about 40%) is assimilated in synthetic processes in the organism, the remainder undergoing further oxidation.

(c) *The metabolism of other substrates.* When the sodium salts of succinic, fumaric and malonic acids were tested as substrates, the curves shown in Fig. 5

Table 4. The effect of various oximes on the metabolism of pyruvic-oxime

(Present in all flasks: 1.0 ml. suspension; 1.0 ml. 0.1M-phosphate buffer, pH 7.2; 0.2 ml. 0.02M-pyruvic-oxime; 0.2 ml. 0.02M-inhibitor and water to total vol. of 3.2 ml. Air. 37°. KOH in centre wells.)

Inhibitor	Oxygen uptake in 45 min. (μl.)	Correction for O ₂ uptake in the absence of pyruvic-oxime and in the presence of the inhibitor (μl. O ₂)	Percentage inhibition of rate of O ₂ uptake
None	120	19	0
Phenylpyruvic acid oxime	110	43	33
D-Arabinose oxime	80	25	46
α-Ketoglutaric acid oxime	98	30	32

were obtained. They indicate that malonate is not metabolized by this organism, succinate after a short lag period, and fumarate but feebly.

no free nitrite, and yet after 2 hr. incubation with the organism this substance consistently gave 5-10 % of its total nitrogen content as free nitrite. Some increase in O₂ uptake is obtained (Table 4), but this is also seen to some extent with the other two oximes which yield no nitrite. This activity is so feeble, however, that it has been classified as approximately zero in considering enzyme substrate specificities. The other two oximes have never yielded any nitrite with this organism.

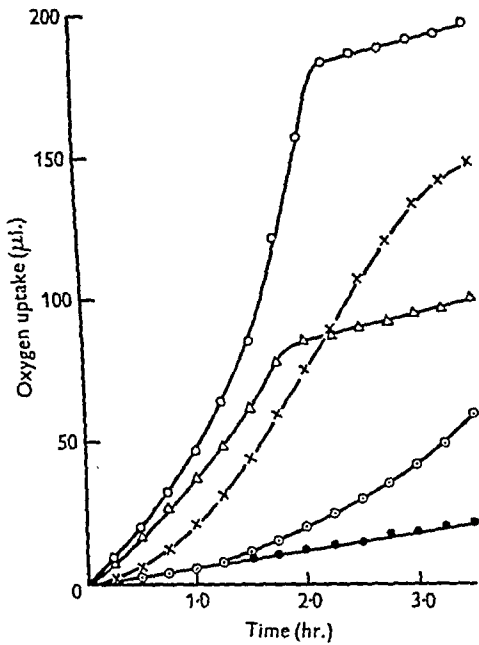


Fig. 5. The oxidation of various carboxylic acids by *Achromobacter* sp. (1) compared with that of pyruvic-oxime. Contents of flasks as in Fig. 1 with the following substrates replacing pyruvic-oxime. ●, water or sodium malonate (0.2 ml. 0.02M); ⊙, sodium fumarate (0.2 ml. 0.02M); x, sodium succinate (0.2 ml. 0.02M); Δ, sodium pyruvate (0.2 ml. 0.02M); ○, pyruvic-oxime (0.2 ml. 0.02M).

(d) The effects of other oximes. The effects of pure samples of the oximes of α-ketobutyric acid, phenylpyruvic acid, α-ketoglutaric acid and arabinose on pyruvic-oxime oxidation were investigated (Quastel *et al.* 1950). The results are shown in Table 4 and indicate inhibitory effects of three of these oximes.

Phenylpyruvic acid oxime solution usually shows

Table 5. Metabolism of oximes by *Achromobacter* sp. (1)

(Conditions as in Table 4)

Substrate added (μmoles)		Oxygen uptake in 60 min. (μl.)	Nitrite formed finally (μmoles)
Pyruvic-oxime	α-Ketobutyric acid oxime		
0	0	30	0
10	0	225	7.39
0	10	117	9.36
10	10	184	16.76

It is evident, therefore, that whilst the oxidation of various oximes to nitrite in soil is confined to those of the α-ketoacids, the ability of isolated organisms to oxidize the oximes is more restricted. Thus *Achromobacter* sp. (1) can metabolize pyruvic-oxime but not (or only very feebly) the oximes of the α-ketoacids mentioned above, although some of these are quite readily metabolized on perfusion through soil. One other oxime is, however, metabolized by this organism, i.e. the oxime of α-ketobutyric acid. Evidence for this is presented in Table 5. Both oximes are readily oxidized, but admixture causes the rate of O₂ uptake to fall between the values given by each oxime alone. This would indicate that the two substrates compete for a single enzyme system. Nitrite formation at the completion of the experiment accounts for 74 % of the added pyruvic-oxime nitrogen and 94 % of the added α-ketobutyric acid oxime nitrogen. In a mixture of the two oximes the nitrite yield is ultimately completely additive.

No period of adaptation seems to be necessary before the oximes are metabolized, and in no manometric experiment has any evidence been obtained which would indicate that *Achromobacter* sp. (1) may become adapted to oxidize any oxime other than those of the α -keto fatty acids.

SUMMARY

1. Oximes of the α -ketoacids (pyruvic, phenylpyruvic, oxaloacetic and α -ketoglutaric) are readily nitrified in soil.

2. The oximes of salicylaldehyde, furil, arabinose, acetone, formaldehyde, acetoacetic ester, and benzoin are inert in soil as nitrite-formers and do not inhibit nitrification of pyruvic-oxime in soil.

3. Among six sulphanilamide derivatives tested, sulphadiazine (0.005%) has the most marked inhibitory action on nitrite formation from pyruvic-oxime in soil. Chloromycetin (1 in 40 000) is highly inhibitory, but penicillin and streptomycin are relatively inert under soil conditions. *p*-Aminosalicylic acid has a small inhibitory action, but nitrourea (0.0033M), which completely inhibits nitrite oxidation in soil, has no inhibitory effect on nitrite formation from pyruvic-oxime.

4. Details are given of the isolation and characterization of organisms in soil and in faeces capable of oxidizing pyruvic-oxime. Three species of organisms, attacking pyruvic-oxime, have been isolated from soil. Two of the species fall into the genus *Achromobacter*; the third is identified as a *Corynebacterium*.

5. Among twenty-two stock cultures of bacteria tested for their abilities to proliferate on a pyruvic-oxime medium, a few gave sparse growth and none formed nitrite.

6. Using washed suspensions of *Achromobacter* sp. (1) it is found that during the oxidation of pyruvic-oxime, the ratio of nitrite production to oxygen uptake remains constant.

7. The results of perfusion and manometric experiments indicate that alanine is not an intermediate in pyruvic-oxime oxidation. Hydroxylamine and ammonia are also unlikely to be intermediates.

8. Suspensions of resting *Achromobacter* sp. (1) oxidize acetate and pyruvate readily, succinate after a short lag period and fumarate very slowly. Malonate is not attacked.

9. The evidence would indicate that pyruvic-oxime is oxidized to pyruvate and nitrite, the pyruvate then undergoing further breakdown. The enzyme system responsible for the oxidation of pyruvic-oxime is termed pyruvic-oxime oxidase. Sodium azide and sodium cyanide markedly inhibit the activity of pyruvic-oxime oxidase.

10. Pyruvic-oxime oxidation by suspensions of resting *Achromobacter* sp. (1) is inhibited by the oximes of α -ketoglutaric acid, phenylpyruvic acid and arabinose. These oximes are apparently either feebly or not attacked by pyruvic-oxime oxidase. The oxime of α -ketobutyric acid is also oxidized by washed suspensions of *Achromobacter* sp. (1), probably by the pyruvic-oxime oxidase.

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ADDENDUM

The Isolation of Bacteria from Soil Perfused with Pyruvic Acid Oxime

By J. H. QUASTEL, P. G. SCHOLEFIELD AND J. W. STEVENSON

Research Institute, Montreal General Hospital, Montreal, and Department of Bacteriology and Immunology, McGill University, Montreal

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Approximately 2 g. of perfused soil 'crumbs' were brought into a finely particulate suspension in 6 ml. of sterile distilled water by gently grinding in a mortar. Larger soil particles were allowed to settle out and the supernatant fluid which contained the smaller particles in suspension was cultured. The culture medium, which contained pyruvic-oxime as the only source of assimilable carbon and nitrogen, was prepared

as follows: K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.2 g.; $MnSO_4 \cdot 4H_2O$, trace; $FeCl_2 \cdot 6H_2O$, trace; Granular Agar (Difco), 15 g.; pH adjusted to 7.0 with NaOH. Dispensed in 100 ml. lots and autoclaved at 120° for 20 min. 0.25M-Pyruvic-oxime (10 ml.) rendered sterile by Seitz filtration was added to each 100 ml. lot of molten agar-salt solution base and plates were poured.

An inoculum of 0.1 ml. of the soil suspension per plate, spread evenly over the surface of the agar, provided cultures in which colonies were, for the most part, discrete. Plates were incubated at 25 and 37°. Despite the fact that perfusion had been carried out at 21° optimal growth was obtained at 37°. There was no selective effect of incubation temperature, plates incubated at 25 and 37° showing the same flora. Incubation for 72 hr. was required to bring out characters by which various colony types might be distinguished one from the other. No new forms appeared on continued incubation beyond this line.

A number of picks of each colony type were grown in pure culture on pyruvic-oxime agar in order to test their ability to attack pyruvic-oxime with the liberation of free nitrite. The test consisted in removing a small square of the agar bearing the growth (0.5 sq.cm.) which was dropped into Ilosvay reagents in a tube. All picks gave strong tests for nitrite. Control squares of sterile pyruvic-oxime agar incubated for the same period of time showed no trace of nitrite. The various picks upon taxonomic study were resolved into three distinct species. It is of interest to note that all of the organisms isolated from the soil on the pyruvic-oxime medium were capable of oxidizing pyruvic-oxime with the production of nitrite. This would tend to suggest a process of selection or adaptation or perhaps a combination of both phenomena during perfusion.

Two of the species isolated from the perfused soil fall into the genus *Achromobacter*; the third has been identified as a *Corynebacterium*. A third species of the genus *Achromobacter* capable of oxidizing pyruvic-oxime with the formation of nitrite has been isolated from a sample of faeces. None of the species which were isolated conforms with species described under the genera *Achromobacterium* and *Corynebacterium* in Bergey's Manual (1948). Due to the unsettled state of classification of species within these genera the organisms isolated have not been given specific designations.

The following are the characteristics of the various species.

Achromobacter sp. (1)

From perfused soil.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0.5 by 1-3 μ . No endospores. Not encapsulated. Motile, possessing peritrichous flagella. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2-3 mm. in diameter, low convex, circular with entire edges, grey, smooth and glistening, translucent, mucoid. Growth in peptone broth turbid with pellicle and sediment, aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H₂S not produced. Indole not formed. Acetylmethylcarbinol not produced. Cannot use salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (2)

From perfused soil.

Morphology. Rods with rounded ends occurring singly and in pairs. Coccoid and oval forms 0.5-1 by 1-3 μ . No

endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 1-2 mm. in diameter, high convex, circular with entire edges, grey, smooth and glistening, translucent. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H₂S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (3)

A third species of *Achromobacter* capable of oxidizing pyruvic-oxime with nitrite production was isolated from faeces and characterized as follows.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0.5 by 1-3 μ . No endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2-3 mm. in diameter, low convex, circular with entire edges, grey to faint cream colour (no pigment on colourless media), smooth and glistening, translucent. Non-mucoid. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates very slowly reduced to nitrites. H₂S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. Acid from dextrose and galactose. No acid from arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

The essential points of difference upon which the separation of the foregoing organisms as distinct species is based are outlined in Table 1.

Table 1. *Distinguishing features of three species of Achromobacter which oxidize pyruvic-oxime*

Test	<i>Achromobacterium</i> sp. (1)	<i>Achromobacterium</i> sp. (2)	<i>Achromobacterium</i> sp. (3)
Motility	+	-	-
Nitrate reduction	Rapid	Rapid	Very slow
Acid from dextrose	-	-	+
Acid from galactose	-	-	+
Use of salts of citric acid as sole source of carbon	-	+	+

Corynebacterium sp.

From perfused soil.

Morphology. Slightly curved rods, 0.5-1 by 2-4 μ . in young culture. 1 by 6-8 μ . in older culture. Palisading and angular arrangement. Pleomorphic on ageing, with club forms. No endospores. Not encapsulated. Non-motile.

Uniformly Gram-positive in young culture tending to variability in older culture with Gram-negative cytoplasm beaded with Gram-positive granules. Not acid-fast.

Cultural characteristics. Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, white, smooth non-glistening surface. Growth in peptone broth: granular sediment with little general turbidity. Aerobic. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates slowly reduced to nitrites. H₂S not produced. Indole not formed. Cellulose not digested. Starch not hydrolysed. Acid from levulose. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Unless otherwise indicated, *Achromobacter* sp. (1) has been used for all the manometric studies.

In order to determine whether or not species of bacteria other than those isolated from the perfused soil might attack pyruvic-oxime with the production of nitrite, a series of laboratory stock cultures which had been maintained on peptone agar were transferred to pyruvic-oxime agar. The results are outlined in Table 2. It is seen that none of a variety of species held in stock possessed, initially at least, the ability to produce nitrite from pyruvic-oxime. Indeed many of the species tested failed to grow on this medium. Growth where it occurred was in all instances rather sparse requiring from 3 days to 1 week of incubation, depending upon the species. The finding that certain heterotrophic organisms may oxidize pyruvic-oxime to nitrite has been confirmed by the recent work of Jensen (1951).

It was felt that adaptation might be a factor in determining the ability of an organism to attack pyruvic-oxime. In order to test this, four selected species, *Achromobacter hartlebii*, *Achromobacter perolens*, *Agrobacterium radiobacter*

and *Bacterium globiforme*, were carried through six successive transfers on pyruvic-oxime agar. On the sixth transfer the two species of *Achromobacter* produced very small amounts

Table 2. *The ability of various stock cultures of bacteria to grow on pyruvic-oxime agar and to produce nitrite from pyruvic-oxime*

Organism	Growth	Nitrite
<i>Achromobacter hartlebii</i>	+	—
<i>Achromobacter perolens</i>	+	—
<i>Achromobacter</i> sp. (isolated from fish)	+	—
<i>Alcaligenes faecalis</i>	+	—
<i>Agrobacterium radiobacter</i>	+	—
<i>Chromobacterium violaceum</i>	—	—
<i>Pseudomonas aeruginosa</i> (2 strains)	+	—
<i>Escherichia coli</i>	—	—
<i>Aerobacter aerogenes</i>	+	—
<i>Klebsiella pneumoniae</i>	+	—
<i>Serratia marcescens</i>	+	—
<i>Proteus vulgaris</i>	—	—
<i>Micrococcus ureae</i>	—	—
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	—
<i>Micrococcus epidermidis</i>	—	—
<i>Micrococcus agilis</i>	—	—
<i>Sarcina lutea</i>	—	—
<i>Corynebacterium segmentosum</i>	—	—
<i>Bacterium globiforme</i>	+	—
<i>Bacillus mesentericus</i>	—	—
<i>Bacillus cereus</i>	—	—
<i>Bacillus megatherium</i>	—	—

of nitrite. *Agrobacterium radiobacter* and *Bacterium globiforme* failed to produce nitrite. It is of interest to note that the two species which gained, presumably by adaptation, the ability to attack the substrate with the liberation of free nitrite were both members of the same genus as the three active species isolated from perfused soil and faeces.

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The Isolation of Androst-16-en-3 α -ol from Women's Urine

By B. W. L. BROOKSBANK AND G. A. D. HASLEWOOD

St Thomas's Hospital Medical School and Guy's Hospital Medical School, London, S.E. 1

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The musk-smelling androst-16-en-3 α -ol, first isolated from swine testes by Prelog & Ruzicka (1944), was obtained from a glucuronide fraction from men's urine by Brooksbank & Haslewood (1949, 1950) and from the urine of women with adrenal cortical tumours by Mason & Schneider (1950). When Brooksbank & Haslewood (1950) tried to obtain this steroid from the urine of women

not showing any endocrine abnormality, they were able to isolate from the musk-smelling fraction only a small amount of a substance melting at about 160°, and therefore different from the androstenol. It has seemed of interest to us to repeat this work in order to settle, if possible, the question of the nature of the musk-smelling constituent of (hydrolysed) urine from normal women.

RESULTS

The work described below shows that the urine examined contained androst-16-en-3 α -ol (approximately 0.2–0.3 mg./l.), as well as pregnane-3 α :20 α -diol (in a concentration of the order of 0.4 mg./l.); these substances were almost certainly present as glucuronides. The previously described substance of melting point about 160° was not again encountered.

DISCUSSION

The urine used was collected from women in whom there was no evidence of endocrine abnormality, and it probably consisted almost entirely of 'follicular' urine, i.e. excreted in the menstrual cycle before the formation of functioning corpora lutea. It is clear, therefore, that in man, the androstenol is truly characteristic of female, as well as of male, urine. It may be of adrenal origin, as suggested by Mason & Schneider (1950) for a female case of adrenal cortical neoplasm. There is no definite evidence to suggest that the testis is involved in its formation by the male, although it was our impression that rather greater amounts were excreted by men than by women. The amount of pregnanediol isolated was about what might have been expected in 'follicular' urine.

METHODS

In general the methods of isolation of Brooksbank & Haslewood (1950) were closely followed.

'Pregnanediol-like glucuronide' (PLG) was estimated as previously described, and the results expressed as pure sodium pregnanediolglucuronide (NaPG). Melting points are uncorrected. Light petroleum was A.R., b.p. 40–60°. Al₂O₃, supplied by Hopkin and Williams Ltd., was neutralized as described by Shoppee (1949).

Isolation and purification of PLG. Urine (191 l.) was collected from healthy women and from patients (in surgical wards) not suffering from endocrine diseases, cancer, kidney or bladder disturbances. All subjects menstruated normally; they were instructed to collect urine only during the first 14 days after menstrual bleeding had ceased. Urine was preserved with thymol-salicylic acid or butanol. It was extracted with butanol and the PLG entrained as described by Brooksbank & Haslewood (1950) in a manner corresponding to the 'concentrated' conditions of the original small-scale estimation (Bisset, Brooksbank & Haslewood, 1948). The total yield of PLG recovered from barium phosphate precipitates was 1.629 g., which was assayed as approx. 50% pure glucuronide (as NaPG). Of the 1.629 g., 96 mg. was used in experiments, kindly carried out by Dr Mary Barber, on bacterial hydrolysis; it was found that this was unsatisfactory, as the extracts apparently contained an inhibitor of glucuronidase activity. An unsuccessful attempt to remove the glucuronidase inhibitor was made by dissolving the remaining PLG (1532 mg. = 789 mg. NaPG) in *n*-butanol/methanol (2:1 v/v) and eluting it from Al₂O₃ (10 g.) in a swiftly moving column

(2 × 3 cm.). The original solvent eluted 283 mg. (27% NaPG), methanol (300 ml.) gave 956 mg. (39% NaPG) and further methanol (approx. 1 l.) gave 147 mg. (48% NaPG). The total solid recovered thus weighed 1.386 g.; it was equivalent to 517 mg. NaPG and hence 789–517 = 272 mg. (34%) of glucuronide (as NaPG) were lost on the column.

Enzymic hydrolysis of PLG. The PLG (517 mg. as NaPG) recovered from Al₂O₃ (above) in 0.1 M-citrate buffer (770 ml., pH 5.2, Kerr, Graham & Levvy, 1948), was treated with 18 200 units of purified calf-spleen glucuronidase (prepared according to instructions kindly supplied by Dr G. T. Mills of the University of Glasgow) dissolved in water (90 ml.). The mixture, together with about 5 ml. CHCl₃, was incubated at 37°. Commercial deoxyribonucleic acid, to a concentration of about 0.3% (w/v), was added after 2 days as a glucuronidase accelerator (Bernfeld & Fishman, 1950) and incubation was continued for a further 2.5 days. The mixture was then diluted with water to about 1 l. and extracted continuously with ether for about 16 hr.; the aqueous portion was finally shaken with two lots of about 200 ml. each of fresh ether. The combined ether extracts were washed with water, dilute Na₂CO₃ solution, water, dried (Na₂SO₄) and evaporated. The residue weighed 247 mg.; it was partially crystalline and had a strong musk-like smell. If this residue had been pregnanediol, it would correspond to about 401 mg. of NaPG; hence about 517–401 = 116 mg. of glucuronide (as NaPG) remained unhydrolysed. Hence, the approximate volume of urine from which the above 247 mg. of hydrolysed material was obtained corresponded to

$$\frac{1532}{1029} \times \frac{401}{789} \times 191 = 91 \text{ l.}$$

Isolation of steroids. The above hydrolysed ether-soluble neutral material (247 mg.) was left for a few days at 0–5° with acetone (about 10 ml.). The separated solid was collected and washed with a little cold acetone. The insoluble material (58 mg.) was crystallized from ethanol/aqueous NaOH and gave pregnane-3 α :20 α -diol (30 mg., m.p. 228–232°, not depressed by authentic material).

Evaporation of the above acetone liquors left a strongly smelling brown gum (189 mg.) which was purified by chromatography as shown in Table 1.

Fraction 4 (75.2 mg.) from column 1 was crystallized from ethanol/aqueous NaOH and gave pregnane-3 α :20 α -diol (5 mg., m.p. 224–227° not depressed by an authentic sample). Hence, the total weight of purified pregnanediol isolated was about 35 mg., i.e. about 0.4 mg./l. of urine.

Fractions 5 (5.8 mg.) and 6 (2.0 mg.) from column 2 were purified by cooling with a little light petroleum in solid CO₂-acetone mixture, decanting and washing the crystalline residue with a little light petroleum similarly cooled. The residues were recrystallized from aqueous acetone, as also was the residue left on evaporation of the light petroleum liquors. The three samples (none of which weighed more than about 1 mg.) thus obtained had melting points as follows: from fraction 5, m.p. 140–142°; from fraction 6, m.p. 136–138°; from the combined light petroleum liquors, m.p. 142–143°. None of these melting points was depressed by mixture with authentic androst-16-en-3 α -ol. The last sample (m.p. 142–143°) was sent to Dr K. Dobriner, of the Sloan-Kettering Institute for Cancer Research, New York, for an infrared spectral examination. Dr Dobriner reported that the absorption taken in the 'finger-print' region (900–1200 cm.⁻¹) of our sample was identical with that of

Table 1. *Chromatography of hydrolysed ether-soluble neutral material from women's 'follicular' urine*

Fraction no.	Eluted with		Eluate	
	Vol. (ml.)	Solvent	Wt. (mg.)	Appearance
Column 1. Hydrolysed material (189 mg.) on Al_2O_3 (2 g.)				
1	40	50% (v/v) Benzene/light petroleum	73.1	Gum
2	40	50% (v/v) Benzene/light petroleum	9.3	Gum
3	40	Benzene	7.8	Gum
4	40	Ether	78.2	Crystalline
5	40	Acetone	10.0	Gum
6	40	Ethanol	10.0	Gum
Total			188.4	
Column 2. Fraction 1 (73.1 mg.) of column 1 on Al_2O_3 (1 g.) in a column (5 x 0.5 cm.)				
1-3	10	Light petroleum	20.4	Colourless oil, slight smell
4	5	Light petroleum	18.6	Reddish oil, musk smell
5	10	Light petroleum	5.8	Crystalline, musk smell
6	10	Light petroleum	2.0	Crystalline, musk smell
7	20	Light petroleum	4.3	Partially crystalline, musk smell
8	10	50% (v/v) Benzene/light petroleum	7.4	Brown gum, smell
9	20	Benzene	12.6	Gum
Total			71.1	

androst-16-en-3 α -ol. Less pure samples of the androstenol were obtained from fractions 7 and 8 of column 2: more of this substance might have been present in fraction 4 from this column. The previously purified substance of m.p. 160–161° (Brooksbank & Haslewood, 1950) was not detected.

SUMMARY

1. Enzymic hydrolysis of a partially purified 'pregnanediol-like glucuronide' fraction from the urine of women in the 'follicular' phase of the menstrual cycle gave a mixture from which was isolated androst-16-en-3 α -ol and pregnane-3 α :20 α -diol.

2. The amount of the latter substance obtained indicated an original concentration of the order of 0.4 mg./l. of the urine, whilst the androstenol might have been present in amounts of 0.2–0.3 mg./l. The musk-smelling androst-16-en-3 α -ol is thus truly characteristic of the (hydrolysed) urine of men and women.

The authors thank Dr K. Dobriner for the infrared spectral identification, also Dr Mary Barber for several experiments on bacterial hydrolysis and Dr F. T. G. Prunty for advice. Without the generous co-operation of Staff and patients at St Thomas's Hospital this work could not have been undertaken.

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

3. IRON IN THE LIVER AND BLOOD OF THE HATCHING CHICK

By W. N. M. RAMSAY

Department of Biochemistry, University of Edinburgh

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It is generally recognized that in many animals the liver stores an important quantity of iron at all ages. Thus, calculations based largely on figures quoted by Brückmann & Zondek (1939) and by Barcroft (1946) show that in both the adult man and the newborn lamb the liver contains roughly one-third of the non-haem iron in the body. In the course of a study which extended over both sexes of many species, Widdowson & McCance (1948) showed that at 20 weeks of age the livers of pullets contained 14.7 mg. non-haem iron/100 g. wet weight, and similar figures were obtained in pullets of 16 weeks by Chapman, Maw & Common (1950). Ramsay (1951) found that during the last few days of incubation some 200 μ g. haem iron disappears from the chick embryo membranes. As there is an apparently corresponding increase in the rate of accumulation of non-haem iron in the embryo body, it seemed at first sight likely that the membrane haemoglobin was being destroyed (bile pigments also appear at this time), and that the non-haem iron liberated was being accumulated as a store. The present experiments were therefore undertaken with a view to finding out whether a significant proportion of the iron was being concentrated in the liver.

As the total amount of non-haem iron in the hatching chick (excluding the 'spare' yolk) is of the order of 350–400 μ g. (Ramsay, 1951), it may be calculated that if the state of affairs is similar to that in the animals already mentioned, the liver should contain more than 100 μ g. non-haem iron. McFarlane & Milne (1934) determined total iron in the liver of the chick at all stages from 9 days after the eggs were set in the incubator until 3 days after the chicks were hatched. In the hatched chick they found the liver to contain 41 μ g. iron, and in the only two livers tested by the method of Hill (1931), that the non-haem iron was 52 and 66% of the total, or roughly 25 μ g./liver. This figure is much less than might be expected, so it seemed important to make a re-investigation of the question. The results described in this paper establish that the analyses of McFarlane & Milne (1934) were correct and that the liver of the chick at hatching is much

poorer in iron than any other liver so far studied, including that of the chicken at later stages of development.

This finding suggested the possibility that many chicks might be at least on the verge of iron deficiency, so that reliable blood-haemoglobin figures for late chick embryos and young chicks might prove interesting. As such figures did not seem to be readily available, total blood iron was determined in as many chicks as possible in the present series. It was felt that the results would be likely to afford a better estimate of haemoglobin concentration than the usual colorimetric methods, because chick blood contains nucleated erythrocytes and is often quite exceptionally lipaemic. It seemed probable that any error resulting from the presence of traces of non-haemoglobin iron in the plasma or the cells would be very much less than the pronounced errors which may be caused by turbidity in any of the simple dilution methods. The work of Rostorfer (1949), on ducks, suggests that only gasometric analyses (impracticable in this case) would give better results than total iron determinations.

EXPERIMENTAL

Newly hatched chicks from the stock of Brown Leghorn hens maintained by the Poultry Research Centre, Edinburgh, were anaesthetized with ether and bled by cardiac puncture, using an oxalated syringe and needle. The chicks were then killed by a blow on the back of the neck. The livers were removed, taking great care not to permit contamination from the 'spare' yolk, which was carefully removed from the abdomen at the commencement of the dissection. The livers were weighed and glass-homogenized (Potter & Elvehjem, 1936) with 2.5 ml. water.

The blood specimens were analysed for total Fe and the liver homogenates for total and non-haem Fe by the methods previously described (Ramsay, 1951). A volume of 0.03–0.05 ml. blood was used for each analysis, and the high catalase activity of both blood and liver made it necessary to heat all specimens to 100° before adding H_2O_2 . Reduction and colour development with 2,2'-dipyridyl were as described by Ramsay (1951). In all three methods the coefficient of variation (s.d. expressed as a percentage of the mean) was of the order of 3%.

RESULTS

Blood iron. The average iron content of thirteen blood specimens was 35.5 mg./100 ml. (range 31.6–38.0, s.d. 2.0 mg./100 ml.). An isolated value of 44.5 mg./100 ml. was not included in the series. A few analyses were made on blood specimens obtained 19 days (three chicks), 22 days (two chicks) and 23 days (three chicks) after the onset of incubation, but the results all fell within the apparent normal limits for the 21st day (mean $\pm 2 \times$ s.d.). They did not suggest that any pronounced trend in haemoglobin concentration is associated with the hatching process.

Liver iron. The results of the analyses on twenty-one livers at 21 days are summarized in Table 1. The total iron averaged 45.4 μ g./liver (range 32.8–85.4, s.d. 12.5 μ g./liver). The percentage of non-haem iron in the total liver iron was 37–69 (mean 52%, s.d. 8.7).

determined in the present work. The two earlier figures were 52 and 66%, and the mean for the present work was 52% (s.d. 8.7%). It is unfortunate that the present author, in a previous paper (Ramsay, 1950) expressed the view that the analyses of McFarlane & Milne (1934) were erroneously quoted. This view was based on analogy with other species, on a series of calculations in the paper of McFarlane & Milne (1934), which purported to show that during the greater part of incubation and after hatching a substantial proportion of the total iron in the body was concentrated in the liver, and on the fact that these authors failed to notice that the chick liver is actually very poor in iron. The present work emphasizes the last point, shows the analogy to be quite false, and demonstrates that the original analyses of McFarlane & Milne (1934) were correctly reported, while the calculations under discussion were erroneous.

Table 1. *Iron in the liver of twenty-one newly hatched chicks*

	Liver wt. (g.)	Total iron		Non-haem iron		
		(μ g./liver)	(mg./100 g.)	(μ g./liver)	(mg./100 g.)	(% of total)
Mean	0.80	45.4	5.7	22.7	2.9	52
Range	0.56–0.94	32.8–85.4	3.8–10.5	14.4–38.4	1.7–4.7	37–69
s.d.	0.108	12.5	1.51	5.9	0.75	8.7

DISCUSSION

Blood iron. The blood-iron figures on these chicks were similar to, but generally slightly higher than, some made on adult birds from the same flock. The danger of direct comparison between birds of such very different ages is real, but it does seem likely that the chicks were not, in fact, seriously deficient in iron.

If it is assumed that the relation between total iron and haemoglobin is the same in the blood of the newly hatched chick as in that of mammals (cf. Rostorfer, 1949), it may be calculated that the observed iron concentrations correspond to haemoglobin concentrations of about 9.5–11.5 g./100 ml. Zorn & Dalton (1937) made haemoglobin determinations on similar material, but their paper unfortunately lacks the technical information which would have made a useful comparison possible. They record striking fluctuations in haemoglobin concentration during and after incubation, but do not state either the method used or the number of analyses on which their conclusions were based.

Liver iron. The total iron, at 45.4 μ g./liver, agrees well with the figure of 41 μ g./liver reported by McFarlane & Milne (1934). Moreover, the two isolated values which they gave for the proportion of non-haem iron in the liver fall within the limits

It is interesting to note that the proportion of non-haem iron in the liver, at 52%, is much lower than the 75% or over which is commonly found in mammalian livers (Brückmann & Zondek, 1939; McCance & Widdowson, 1940; Scott & McCoy, 1944). It might be thought that this difference was an analytical one, as the chick livers were not washed or perfused to remove blood; they were merely dabbed with filter paper. These livers, however, are rather fatty (20–22% ether-soluble material; Entenman, Lorenz & Chaikoff, 1940; A. K. Lough, private communication), and contain little blood. Moreover, the proportion of non-haem iron was not less than usual in livers which contained much haem iron (and might therefore have been contaminated with larger volumes of blood). The difference is therefore probably a real one, and would be expected from the knowledge that at this stage the chick liver is not carrying a large store of non-haem iron.

Other chicks analysed at the same time gave figures for the quantity and distribution of iron similar to those reported in the previous paper (Ramsay, 1951). Calculations based on the figures in the present work and on values taken from the graphs given by Ramsay (1951) show that the non-haem iron in the liver amounts to no more than 2.0–2.5% of the total iron in the body or 6–8% of the non-haem iron. Recalculation from the analyses of McFarlane & Milne (1934) shows their figures to

be in good agreement with the present ones in this respect also, except that they did not make determinations of non-haem iron in the whole chick. This state of affairs may be in part a reflexion of the small size of the liver, which at hatching amounts to only 2-3 % of the body weight, while according to Hamilton, Boyd & Mossman (1945) the human liver at birth may be 5 % of the body weight. The difference in proportion of functional tissue weight may be very much greater than these figures suggest because, as has already been noted, fully one-fifth of the wet weight of the chick liver consists of ether-soluble material. This particular problem in comparative biochemistry would seem to be an ideal one for attack by the method advocated by Vendrely & Vendrely (1949) and Davidson & Leslie (1950), in which deoxyribonucleic acid determinations are made and the number of cells calculated from the results with the aid of certain fundamental assumptions.

The question arises whether iron normally stored in the liver is displaced in the hatching chick to other sites, or whether a very large proportion of the non-haem iron in the body is to be regarded as belonging to the 'parenchymatous iron' fraction (Hahn & Whipple, 1936) which is not believed to be readily available for haemoglobin formation. Neither the kidney analyses of McFarlane & Milne

(1934) nor a few spleen analyses made in this laboratory suggest that these organs usurp the function of the liver in respect to iron storage. The idea is tempting that the organism does not develop such functions until the 'spare' yolk is exhausted. This does not happen until about a week after the chick is hatched.

The problem remains of the fate of the iron which may be liberated by haemoglobin catabolism towards the end of incubation. Whatever may be the solution, it is certain that iron liberated in this way is not 'stored' in the sense in which that word is usually used in relation to iron metabolism.

SUMMARY

1. Total blood iron in thirteen newly hatched chicks was 35.5 (S.D. 2.0) mg./100 ml.
2. The livers of twenty-one newly hatched chicks contained only 45.4 (S.D. 12.5) μ g. total iron and 22.7 (S.D. 5.9) μ g. non-haem iron.
3. It is concluded that the liver of the newly hatched chick contains no appreciable store of iron.

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Studies in Rhodopsin

4. PREPARATION OF RHODOPSIN

By F. D. COLLINS, R. M. LOVE AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

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The history of visual pigments has been reviewed on numerous occasions—see Collins & Morton (1950) and Wald (1951). In recent years the methods used in preparing solutions of rhodopsin have involved at least partial separation of rod outer segments. If retinas are shaken vigorously in saline or other suitable suspending medium the rod outer segments become detached. Various methods have been used for separating these 'rods' from the remainder of the retinal suspension. Lythgoe (1937) poured the suspension through fine wire gauze and showed that the rods, pigment granules and a few isolated cells passed through, but that the main mass of nuclear and fibrous material was held back. Krause & Sidwell (1938) centrifuged the retinal suspension and found that the rods formed a layer on top of the other retinal fragments which could then be scraped off. Saito (1938) used 40–45 g. sucrose/100 ml. water as the suspending medium and found that on centrifuging, the rods remained in suspension. The rods could be thrown down when diluted with sufficient saline. The method has been used by Collins & Morton (1950), by Bliss (1950) and by Wald (1951). In addition, Wald (1949) has often simply scraped the rods off the retina with a fine spatula or forceps.

The purity of solutions of rhodopsin prepared from rods isolated by any one of the methods described above varies greatly. With frog retinas it is easier to obtain pure solutions than with cattle retinas. It was the purpose of the present work to investigate more fully the reasons for these differences and to develop improved techniques applicable to cattle retinas which can be obtained from abattoirs in large numbers. Comparatively large amounts of cattle rod outer segments would greatly facilitate future work.

EXPERIMENTAL

Solutions

Potash alum $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$, 4% (w/v) in water.

Digitonin solution. 1 or 2% (w/v) in water; prepared by heating the mixture of digitonin and water to boiling until solution occurs and then quickly cooling.

Buffer solution, pH 9.3. $Na_2B_4O_7 \cdot 10H_2O$, 19 g./l.

Formaldehyde (neutral). A 40% (w/v) solution of formaldehyde was shaken with $MgCO_3$, allowed to stand overnight and filtered.

Animals

Eyes were obtained from freshly killed cattle and transported from the local abattoir to the laboratory in closed tins. The dissection, carried out in red light, has been described by Collins & Morton (1950).

The frogs used were *Rana esculenta*.

Absorption measurements

All measurements were made with a Beckman photoelectric spectrophotometer. A 1 cm. cell was used. The compensating cell contained a solution of digitonin and buffer equivalent in strength to that used for the rhodopsin solutions. The cells were filled in the dark room (using red light), placed in the cell holder and covered with a black cloth. They were then carried to the Beckman and placed in position in the light-tight cell space. Although rhodopsin solutions are sensitive to light no noticeable photodecomposition took place during measurements.

Criteria of purity

The spectroscopic purity of rhodopsin solutions has been assessed as follows. The ratio of the extinction at wavelength λ to that at λ_{max} (about 500 m μ .) has been designated P_λ . It is found that in the presence of absorbing impurities P_λ is higher than that for pure rhodopsin. Hence as the degree of purity increases P_λ will tend to a constant low value. Usually P_λ has been measured at 400 m μ ., a minimum in the rhodopsin curve (cf. Collins & Morton, 1950). The lowest value so far obtained for P_{400} is about 0.25. As rhodopsin solutions have an absorption maximum near 275 m μ . due to the presence of protein (tyrosine and tryptophan) and also some nucleotide absorption near 260 m μ . (Collins, Love & Morton, 1952) it has been found useful to record P_{275} in some cases.

RESULTS

Preliminary investigations

Separation of rods by scraping. Wald (1949) separated rods by carefully scraping the surface of the retina with a fine spatula. This method was investigated.

Cattle retinas were spread out flat on a piece of filter paper with the rods uppermost. The surface was then gently stroked with a coarse hair brush, a spatula having been found useless, and the brush shaken at frequent intervals in saline solution. The saline suspension, when examined microscopically, was seen to contain large fragments of tissue, rods and blood cells. In another experiment a fine camel-hair brush was used and microscopical examination revealed a suspension of rods and blood cells only. In both

cases a sugar separation (see next section) was subsequently carried out, the rods hardened in alum and finally extracted with aqueous digitonin solution. The solutions yielded values of P_{400} of 0.53 and 0.68 respectively.

These results and the extra time required made this method unsuitable for cattle retinas.

Crude rod suspensions. These can be obtained by shaking a number of retinas in saline or sucrose solutions and pouring through a fine wire gauze (60-mesh). The resulting suspension contains most of the rods but is heavily contaminated with other tissue fragments. Lythgoe (1937) pointed out that frog retinas do not fragment during shaking, apart from releasing the rod outer segments, but cattle retinas as a whole appear to break up into small pieces. This we confirmed. Frog and cattle retinas were shaken vigorously in saline. The suspension of frog 'rods' was almost uncontaminated by other tissue fragments, but the suspension of cattle rods contained many blood cells and a high proportion of unwanted tissue and melanin pigment granules. The frog rod outer segments were much the larger.

Separation of rods by Saito's method

Saito's method, as modified by Collins & Morton (1950), was used at first. However, the purity of cattle rhodopsin solutions obtained by this method was very variable and the need for improvement soon became apparent.

The original method was as follows. The cattle retinas, dissected in red light, were shaken vigorously in 1.32M-sucrose for 30 sec. using 15 ml. sucrose solution per twenty-four retinas, and poured through the fine wire gauze. The resulting suspension was centrifuged at 1600 g for 15 min. At the end of that time it could be seen that the fibrous part of the retina, blood cells and melanin were thrown to the bottom of the tube, while the rods remained suspended in the supernatant. This was decanted, diluted with 2-3 vol. of 0.9% (w/v) saline and centrifuged until clear. The brilliant scarlet precipitate was hardened in 4% (w/v) alum solution for 1 hr. The rods were then centrifuged down and washed once with saline, and finally extracted for 1 hr. with 1 ml. of 1% (w/v) digitonin solution and centrifuged. The clear supernatant was mixed with 1 ml. of buffer solution (pH 9.3), recentrifuged, and the absorption spectrum measured.

The following variables were investigated:

(i) The effect of pH on the digitonin extraction. The least amount of contaminating impurities were extracted at an acid pH, but a fine precipitate tended to form which could not be centrifuged down. Alkaline extracts were very impure. Hence a neutral extractant was employed.

(ii) The concentration of sugar seemed unimportant between about 1.0 and 1.6M, but below 0.88M the rods sank to the bottom and formed a layer on the debris, while above about 1.6M the whole brei floated.

(iii) Re-extraction of the retinal debris with 1.32M-sucrose yielded a less pure fraction. More rods were obtained, but with four successive 'sugar extractions' the purity deteriorated thus: P_{400} = 0.446, 0.53, 0.733 and 0.858.

(iv) The duration of the alum treatment is apparently unimportant provided it exceeds 30 min. If the rods are left for periods greater than 24 hr. at room temperature there is a slow loss of rhodopsin.

(v) The extraction by means of digitonin solution is nearly complete in 1 hr., but thereafter increasing amounts of impurities go into solution.

Attempts to remove impurities from rods. Rods separated by Saito's method were extracted with an alkaline buffer (pH 9.3) followed by an acid one (pH 4.1). The rods were then hardened in alum and extracted as usual; P_{400} = 0.477. In other experiments the rods were extracted with 0.1% Dispersol A (an Imperial Chemical Industries Ltd. detergent); untreated rods gave a value of P_{400} = 0.783, whilst after treatment P_{400} became 0.565. Further experiments showed that only very heavily contaminated rod preparations were improved.

The following organic solvents were tried: phenol, acetone, amyl alcohol, pyridine, cyclohexane, ethyl acetate and ethyl butyrate. All except the last bleached the rhodopsin immediately—ethyl butyrate did so in about 24 hr.

It was noticed that rods spun down after having been separated were contaminated with many black particles. Attempts were made to separate these small particles using a fine spatula. A typical experiment was as follows: the rods were divided into two portions: (1) was used as a control and (2) was separated into (2a) rods relatively free from black particles and (2b) rods contaminated with black particles. The values of P_{400} were respectively 0.282, 0.312 and 0.362. In another experiment the 'clean' rods gave P_{400} = 0.30 and contaminated rods gave P_{400} = 0.37. The conclusion to be drawn from this experiment was that even the 'cleaned' rods gave less pure solutions than those obtained from untreated rods.

Treatment with formaldehyde. Although ordinary formaldehyde solutions (40%, w/v) at once destroyed rhodopsin it was found that a neutralized solution (i.e. left to stand with magnesium carbonate overnight) did not. It was found that solutions of rhodopsin prepared from rods treated with formaldehyde had a value of P_{275} lower than had been obtained previously and that 10 min. in formol was the optimum time of treatment (Tables 1 and 2). Good results were obtained by combining formol treatment with differential centrifugation (see below) in winter and spring, but the results in summer were unsatisfactory (Table 2). The purest cattle rhodopsin solution obtained by this method had P_{400} = 0.236, P_{275} = 2.05 at pH 9.3 (Fig. 2). Later experiments have shown that it is better to treat with alum before the formol treatment.

Attempts to remove impurities from rhodopsin solutions. Various attempts were made to precipitate the rhodopsin from its digitonin solution in the hope that impurities would be left in solution. Acetone was tried first; this solvent destroys rhodopsin at room temperature but not at 0°. However, it was found that all the protein present was precipitated and that no purification resulted. Addition of acetone in steps might be more successful if combined with facilities for low temperature centrifugation.

Sodium sulphate and magnesium sulphate and sodium chloride were next tried, but did not result in any improvement when the precipitated rhodopsin was redissolved. Ammonium sulphate was more successful.

Table 1. *Effect of duration of treatment of rods with formaldehyde on purity of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for various periods and then treated with alum and extracted with digitonin solution as usual. The data refers to the absorption spectra of the resulting solutions.)

Time in formol (min.)	P_{400}	P_{275}	$E_{500m\mu}$
First experiment			
0	0.322	3.3	0.251
10	0.318	2.45	0.277
20	0.322	2.55	0.192
40	0.350	2.88	0.148
17 hr.	About half of the rhodopsin destroyed		
Second experiment			
0	0.330	3.64	—
5	0.295	2.45	—
10	0.295	2.11	—
15	0.300	2.18	—

Table 2. *The effects of treating rod outer segments with formaldehyde solution on purity and yield of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for 10 min., then with alum for 1 hr. and extracted as usual with digitonin solution. The data refer to the absorption spectra of the resulting solutions.)

Solutions prepared in the winter						
	Formol treatment			No formol treatment		
	P_{400}	Yield*	P_{275}	P_{400}	Yield*	P_{275}
Mean	0.303	0.056	2.80	0.293	0.066	3.84
Standard error	0.0089	0.0075	0.15	0.0096	0.0064	0.33
No. of samples	19	18	19	13	12	14
Range	0.236-0.39	0.02-0.14	2.05-4.9	0.217-0.34	0.03-0.10	2.8-7.2
Solutions prepared in the summer						
	Formol treatment		No formol treatment			
	P_{400}	P_{275}	P_{400}	P_{275}		
Mean	0.515	6.9	0.324	4.1		
Standard error	0.0605	0.87	0.017	0.29		
No. of samples	11	10	11	9		
Range	0.26-0.93	2.9-11.3	0.27-0.44	2.95-5.9		
* Yield = $\frac{(\text{Extinction at } 500 \text{ m}\mu, \text{ due to rhodopsin}) \times (\text{vol. of soln.})}{(\text{No. of retinas})}$.						

Rods from fifty cattle eyes were extracted three times with 1 ml. of 0.5 % digitonin solution and the combined extracts were mixed with an equal volume of buffer, pH 4.5. The resulting solution had P_{400} = 0.575, P_{275} = 7.7. On adding increasing amounts of solid ammonium sulphate four fractions were obtained. The first contained most of the rhodopsin, P_{275} and P_{400} both being lower than in the

original solution. The second fraction contained a little rhodopsin of low optical purity. Fractions 3 and 4 contained no rhodopsin, but had maxima near 270 m μ . These results showed that rhodopsin solutions contained irrelevant material absorbing at 275 m μ . and that some of this impurity could be removed by fractional precipitation with ammonium sulphate. Better results were obtained later by another method.

Modifications of Saito's method

Differential centrifugation. Rods from twenty-four ox eyes were prepared by Saito's method. The sugar suspension was diluted with 3 vol. of saline and centrifuged for 2 min. only at 1200 g. The precipitate was treated with alum and the supernatant cleared by centrifuging for 15 min. at 1800 g, and *this second precipitate was also treated with alum.* When the precipitates were extracted, the values of P_{400} were 0.28 and 0.38 respectively. The value of P_{275} for the first solution was 2.29. It was found that for this method to be effective the eyes had to be dissected and extracted very soon after death. Another disadvantage was the low yield—less than 20 % of the total rhodopsin/retina.

The sugar gradient method. The combination of differential centrifugation and extreme freshness of the eyes in the Saito method produced many solutions of cattle rhodopsin purer than had ever been obtained before, but in view of the low yield and the difficulty experienced in obtaining sufficiently fresh eyes a new modification was developed and is described below.

Retinas are shaken vigorously with about half their volume of 0.9% (w/v) saline in order to detach the rod outer segments. The mixture is stirred into a piece of 60-mesh brass gauze shaped in the form of a hollow until all the liquid has passed through, whereupon the retinal debris is transferred to a glass tube and shaken again with saline. This treatment is done four times, after which the retinal fibrous mass becomes considerably reduced in bulk and is usually colourless. The resulting suspension contains tissue fragments, blood, melanin and rods. It is transferred to a centrifuge tube, which should not be more than three-fifths filled by it. Saturated sucrose solution—greater than 2.0M—is now carefully poured down the side of the tube so as to form a lower layer of about half the volume of the saline

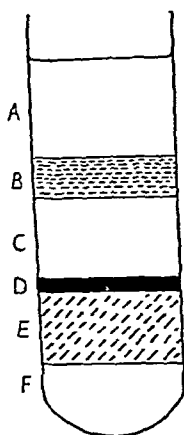


Fig. 1. This diagram shows the disposition of the various tissue fragments after being centrifuged in sucrose solution in which a density gradient had been established as described in the text (p. 295). A, pale opalescent solution containing some protein; B, dense, deep scarlet, layer of rod outer segments; C, faintly pink opalescent layer containing a few rod outer segments and some small tissue fragments; D, dense, dark red, layer containing mostly red blood cells; E, densely packed layer, containing the greater bulk of the disintegrated retina as well as melanin granules; F, colourless, almost saturated, sucrose.

suspension. The next part of the process requires practice. A flat-ended glass rod is used, and with it the saline-sugar interface is stirred. The object is to produce a gradient of concentrations of sucrose down the tube, ranging from zero, i.e. saline only, at the top, through gradually increasing concentrations, to saturated sucrose at the bottom. The tube is then centrifuged at 1800g for 15–20 min. On removing the tube from the centrifuge, all the components of the suspension are found to have accumulated in their own pycnotic level.

Fig. 1 shows a diagram of an actual separation. The various zones are sucked off using a tube drawn out at the end and turned up so as to resemble a crochet hook. The rods are diluted with 2–3 vol. of saline and centrifuged down.

It was found that sucrose solutions in a series of strengths from 0.76 to 1.17M at 0.06 intervals could, by careful pipetting, be placed one on top of another in a centrifuge tube without mixing appreciably.

The strongest ones were, of course, on the bottom, and the boundaries between the solutions could be clearly seen. Their positions were then marked. The crude saline suspension of rods was pipetted on top of them all and the whole centrifuged. It was found that cattle rods are isopycnotic with 0.88 (± 0.15)M-sucrose, while the blood cells, etc., come to rest in sucrose of greater than 1.0M. This being so, it was hoped that, by making a series with a deep 0.94M layer, the rods could be separated a long way from the blood cells. The idea was eventually abandoned, however, since once the technique had been learned the stirring method was far less tedious. The rods from frogs appear to be denser than cattle rods.

Table 3. Variations in the purity and yield of rhodopsin solutions from rod outer segments prepared by the sugar gradient method

(Cattle rod outer segments were separated by flotation in sucrose solution with a density gradient. The isolated rods were treated with alum and extracted with digitonin solution. The data refer to the absorption spectra of the resulting solution.)

	P_{400}	P_{775}	Yield*
Mean	0.293	3.83	0.212
Standard error	0.0061	0.13	0.035
No. of samples	16	14	11
Range	0.26–0.34	3.1–4.5	0.05–0.49

* Yield

$$= \frac{(\text{Extinction at } 500 \text{ m}\mu. \text{ due to rhodopsin}) \times (\text{vol. of soln.})}{(\text{No. of retinas})}$$

If the rod preparation was not very pure, then a repetition of the gradient process effected an improvement, but further treatments only worsened the preparation, due, no doubt, to excessive handling of the rods. In any second purification, the top layer was usually water-clear, but a little retinal tissue could be seen under the rod 'band'.

Table 3 shows that the yield of rhodopsin per retina is greatly improved and that the values of P_{400} indicate fairly good purity.

For these procedures it was unnecessary to use extremely fresh eyes. On one occasion 130 eyes were treated in one day and, although the time taken for dissection and treatment was considerable, the value of P_{400} for an extract of some of the resulting rods was 0.30.

Microscopic examination of rod suspensions in the course of various treatments

No staining or fixing was used.

(a) Sugar separation. (i) Crude rod suspension. Rods were mostly intact with many red blood cells and other, not easily distinguished, tissue fragments present. (ii) After a sugar separation. Some rods were deformed, but the majority were unchanged. Very few other tissue fragments were present. (iii) After another sugar separation. Very few intact rods remained.

(b) Effect of time on a rod suspension in 0.9% (w/v) NaCl. (i) Fresh—rods remained intact. (ii) 0.5–1 hr.—some changes were noticed. (iii) 1–2 hr.—many deformed and broken rods were seen. (iv) 3 or more hr.—very few intact rods remained.

(c) Effect of various reagents on rod suspensions. (i) Formaldehyde (40% neutralized)—the rods were seen to curl up forming almost complete circles and sedimented readily. (ii) Digitonin solutions—the rods were broken up completely. (iii) Alum solutions (4%, w/v)—the rods were broken up completely and sedimented quickly. (iv) A solution containing 2% formaldehyde and 0.9% KCl—the same changes occurred as described under (b) but a little more slowly.

DISCUSSION AND CONCLUSIONS

Separation of rod outer segments

The separation of rod outer segments from the other tissue fragments present in the initial crude suspension depends on two factors, the size and the density of the rods. The rods are fairly large (about $3 \times 30 \mu$) and sediment rapidly in water even if the centrifugal force is as low as 600 g. In this respect they are comparable to red blood cells and nuclei. However, the rods differ from blood cells and nuclei in one important property, they are less dense and will float in 0.88M-sucrose (sp.gr. = 1.114).

If these were all the facts then the methods described would separate rods from all tissue fragments except (a) particles isopycnotic with rods, and (b) particles so small that, at the centrifugal forces used (about 1800 g), they are removed too slowly from the rod zone. A second separation should enable the majority of small particles not isopycnotic with the rods to be eliminated. This presupposes that rod outer segments are stable. However, microscopic examination reveals quite clearly that they are not and that they fragment. This explains why a repetition of a sugar separation does not result in any great improvement. As the rods break up greater centrifugal forces would become necessary to effect a separation. This was not considered practical, and the writers prefer one sugar separation performed as quickly and as efficiently as possible. Subsequent treatments with alum and neutralized formaldehyde yield solutions which are very pure.

A recent study, using the electron microscope, of rod outer segments by Sjöstrand (1949) indicates that rods are composed of several thousand disks each about 3μ thick. He records the fact that rods when broken up yield fragments which may contain a few disks or many hundreds. This is in good agreement with our own observations and reveals still more clearly the difficulties encountered in attempting to separate rod outer segments.

The effect of this fragmentation of rods will be manifested in two ways. The first will be the in-

creased difficulty of effecting an efficient separation from other tissue fragments. The second and perhaps more serious will be the presence of submicroscopic particles (fragments containing only a few 'disks') which will not be centrifuged out after the digitonin extraction. This will make the final rhodopsin solution turbid.

Characteristics of pure (?) rhodopsin

In the absence of any precise chemical criterion of purity spectroscopic ones are used instead. A value of P_{400} below 0.24 to 0.26 has not yet been obtained (cf. Wald, 1951). Similarly, a value of P_{275} of less than 2.05 has not been obtained. The curve shown in Fig. 2 represents such an absorption curve and it is worth while to examine it in detail.

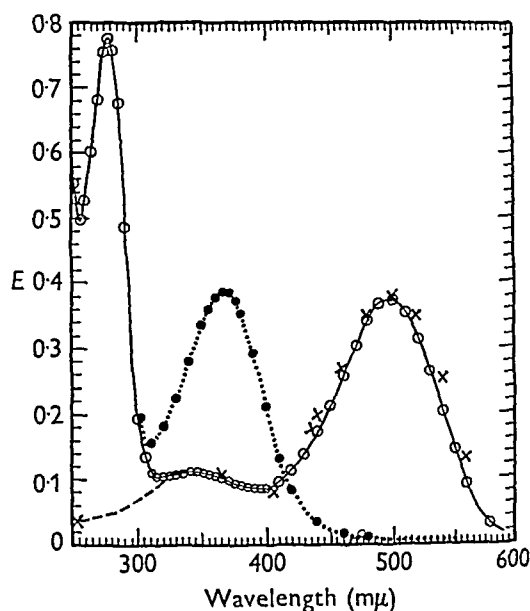


Fig. 2. —○—○—, absorption spectrum of a very pure solution of cattle rhodopsin, pH 9.2; × × ×, relative photochemical efficiencies. $[(\epsilon_{\lambda} \times \gamma_{\lambda})/\epsilon_{500 \text{ m}\mu} \times \gamma_{500 \text{ m}\mu}]$ multiplied by a factor so that the two curves correspond at 500 mμ; after Schneider *et al.* (1939). ..●...●..., absorption spectrum of bleached solution (cf. Collins & Morton, 1950); ----, extrapolation indicating probable absorption of the rhodopsin chromophore below 320 mμ.

In Fig. 2 it is shown that a very close correspondence exists between the rhodopsin absorption curve, and the relative photochemical efficiency curve of Schneider, Goodeve & Lythgoe (1939). The last curve predicts a small peak near to 350 mμ, which is in fact found. It also predicts that at 254 mμ the absorption of the rhodopsin chromophore should be only about 6% of that at 500 mμ. A plausible extrapolation for the absorption due to rhodopsin chromophore is shown in Fig. 2 by means of dashes. The difference between this and that actually found

will be due to the protein containing tyrosine and tryptophan and can be calculated as follows. If E_{280} represents the total extinction at 280 m μ . after subtracting the absorption due to the rhodopsin chromophore, and E_{290} is the corresponding value at 290 m μ . then

$$E_{280} = x + y,$$

$$E_{290} = 0.448x + 0.710y.$$

Here x = extinction at 280 m μ . due to tyrosine and y = extinction at 290 m μ . due to tryptophan, both at pH 9.2. (Collins & Morton, 1950, give similar equations; the difference is due to the fact that a purer sample of tryptophan was obtained for the present work.)

animal proteins is of the same order; casein (6.6 and 1.2), cattle fibrin (6.5 and 3.0), haemoglobin (3.2 and 1.3) serum albumin (4.8 and 0.5) and serum globulin (6.7 and 2.3).

It should be pointed out that the conclusion that the only absorbing materials present are the rhodopsin chromophore, tyrosine and tryptophan does not exclude small amounts of phenylalanine, nucleotides or other unidentified materials absorbing in the region 250–300 m μ .

It is not yet possible to say if the rhodopsin solutions now obtainable are 'pure', but they are in very close agreement with the results obtained by Wald and his co-workers (Wald, 1951). It seems likely that any contaminants present show no

Table 4. *Analysis of the ultraviolet absorption of a rhodopsin solution*
(Solution in 1% (w/v) aqueous digitonin and borate buffer, pH 9.2.)

λ (m μ .)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
300	0.199	0.076	0.123	0.085	0.052	0.137	-0.014
290	0.491	0.058	0.433*	0.127	0.305	0.432	+0.001
285	0.681	0.053	0.628	0.214	0.370	0.584	+0.044
280	0.764	0.051	0.713*	0.283	0.430	0.713	0.000
275	0.753	0.047	0.706	0.297	0.415	0.712	-0.006
270	0.681	0.044	0.637	0.252	0.398	0.650	-0.013
265	0.600	0.042	0.558	0.213	0.344	0.557	+0.001
260	0.529	0.041	0.488	0.188	0.282	0.470	+0.018
255	0.498	0.040	0.458	0.235	0.216	0.451	+0.007
250	0.552	0.040	0.512	0.382	0.164	0.546	-0.034

Extinction due to: (1) Actual rhodopsin solution.

(2) Rhodopsin chromophore.

Extrapolation making the assumptions mentioned in text (p. 297).

(3) Difference between (1) and (2).

(4) Calculated absorption due to tyrosine.

(5) Calculated absorption due to tryptophan.

(6) Sum of (4) and (5).

(7) Difference between (3) and (6).

* (4) and (5) were calculated from these figures using the equations given in the text (p. 297).

In Table 4 is set out the results of an analysis of the curves shown in Fig. 2. The absorption due to tyrosine and tryptophan at 280 m μ . has been calculated and, from the curves of pure tyrosine and tryptophan, the absorption due to these substances has been calculated at other wavelengths. When this absorption, calculated to be due to tyrosine and tryptophan, is subtracted from the protein curve (= rhodopsin less absorption due to the rhodopsin chromophore) the differences are small and are both positive and negative. It may be concluded that, within these limits, the absorption of rhodopsin can be accounted for in terms of the rhodopsin chromophore, tyrosine and tryptophan. If one assumes the data given by Collins & Morton (1950) for the $E_{1\%}^{1\text{cm}}$ of rhodopsin (= 6.6 at 500 m μ .), tyrosine (= 78 at 280 m μ . at pH 9.2) and tryptophan (= 262 at 280 m μ . at pH 9.2) the approximate amounts of tyrosine and tryptophan as a percentage of the dry weight of rhodopsin are 6 and 3 respectively. The percentage of tyrosine and tryptophan in other

absorption in the region 250–700 m μ . and that the solutions are sufficiently pure for chemical analyses to be meaningful.

SUMMARY

1. Various methods for the separation of rod outer segments from cattle retinas have been investigated, attention being paid to the optical purity of the rhodopsin solutions obtained by extraction of the rod outer segments.

2. A new method has been developed depending on the fact that cattle rods have the same density as 0.88M-sucrose. The rods are separated by centrifuging them in a sucrose solution in which a density gradient has been established.

3. Various treatments of the isolated rods have been tried in order to eliminate impurities. The best results have been obtained by treating with 4% (w/v) alum solution for 1 hr. followed by 40% (w/v) neutralized formaldehyde solution for 10 min.

4. The effects of these various treatments on the appearance of the rods has been followed microscopically.

5. Very pure cattle rhodopsin solutions have been obtained showing maxima at 498, 345 and 275 m μ . The last band can be accounted for by the presence of tyrosine and tryptophan only. Making

some plausible assumptions, rhodopsin appears to contain 6% tyrosine and 3% tryptophan on the dry weight of the protein.

We would like to thank the Medical Research Council for grants. One of us (F.D.C.) participated in this work as a holder of an Imperial Chemical Industries Research Fellowship.

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Estimation of Protamine and Insulin in Protamine Zinc Insulin

By F. A. ROBINSON AND KITTY L. A. FEHR

Research Division, Allen and Hanburys Ltd., Ware, Hertfordshire

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In a recent publication, Franklin & Quastel (1950) reported that mixtures of proteins could be separated into their individual components by paper chromatography using solutions of sucrose or sodium potassium tartrate for development. The difficulty of locating the position of the protein zones was overcome by adding haemin to the protein mixture and then streaking the paper with a solution of benzidine and hydrogen peroxide. Jones & Michael (1950) separated proteins on columns of cellulose by development with buffer solutions containing ammonium sulphate and located the position of the individual components by treatment with suitable dyestuffs which stained the zones occupied by the proteins, but did not give coloured spots with amino-acids or peptides. More recently, Papastamatis & Wilkinson (1951) used bromothymol blue and tetrabromophenolphthalein ethyl ester to indicate the position occupied by proteins in paper chromatograms. The publication of this last paper prompts us to report our own work on the chromatographic separation and estimation of protamine and insulin.

The initial experiments on the separation of insulin and protamine by paper chromatography were carried out with strips of Whatman no. 1 filter paper 2 cm. wide, using 10 μ l. spots of a solution containing 1% protamine sulphate and 1% insulin (w/v). The strips were developed in a downwards direction in the usual way according to the method of

Consden, Gordon & Martin (1944) with the phases of a mixture (by vol.) of *n*-butanol (40%), glacial acetic acid (10%) and water (50%), a mixture generally used in the paper chromatography of amino-acids. Solway purple, as recommended by Jones & Michael, was used for indicating the position of the protein bands. Two bands were formed, one due to protamine at the point of application and the other due to insulin, some 6 cm. below the point of application; the solvent front travelled about 25 cm. The separation was not entirely complete, however, and a number of other solvent mixtures were tried. Better results were eventually obtained with the upper phase obtained by equilibrating a mixture of *n*-butanol and glacial acetic acid (3:1 by vol.) with an equal volume of water. This resulted in the formation of two well-defined bands with R_f values of 0 and 0.43 respectively, and this mixture was used in all subsequent work.

The estimation of substances separated by paper chromatography often presents considerable difficulty, although fairly satisfactory methods of estimating individual amino-acids on paper chromatograms have recently been described. It occurred to us that a protein might be estimated with a fair degree of accuracy by the method of retention analysis described by Wieland & Fischer (1948). These workers showed that if a spot of an amino-acid solution was placed near the edge of a

sheet of filter paper and the paper then dipped into a solution of a cupric salt, the latter was drawn up by the paper and cupric ions became uniformly distributed throughout the paper except in the area immediately above the amino-acid spot. In this region no copper could be detected on subsequently spraying with a suitable indicator, because the amino-acid retained the copper ions in the form of a complex, thus preventing them from passing further up the paper. The area of the white copper-free wedge formed above the spot was proportional to the amount of amino-acid present. It was thought possible that the same principle could be applied to the estimation of a protein using instead of a copper salt a solution of a dyestuff with an affinity for the particular protein concerned.

To test this hypothesis spots of different sizes of solutions of insulin, protamine sulphate, blood albumin and edestin were applied to rectangular sheets of filter paper, and the filter papers dried and dipped into solutions of Solway purple or erythrosine. The dyes were allowed to rise about 5 cm. above the protein spots. In every instance a wedge-shaped area above the protein spot remained white and the areas of these wedges were approximately proportional to the amount of protein present.

Attempts were then made to apply retention analysis to mixtures of protamine sulphate and insulin after separation by paper chromatography. A spot of the solution to be tested was applied at one corner of a rectangular sheet of filter paper. The paper was developed along the longer axis with the aqueous butanol-acetic acid mixture and dried. It was then cut in such a way as to separate the protamine which remained at the point of application from the insulin spot which had moved about 10 cm. away from it. Both halves of the paper were then dipped in a dye solution until the dye had risen about 5 cm. above the protein spot. Estimates of the amount of protamine calculated from the area of the unstained wedge above the protamine spot were in fair agreement with the amount of protamine known to be present, but it was impossible to estimate the amount of insulin in a similar manner because the insulin spot, in contrast to the protamine spot, was very diffuse, marked 'tailing' generally having occurred. All attempts to sharpen the insulin zone so as to make it amenable to retention analysis have been unsuccessful.

At this stage we learnt that Dr W. Dickinson had used bromocresol green for indicating the position of different protein bands in paper chromatograms of crude insulin solutions and had succeeded in eluting the insulin bands from such paper chromatograms by means of a borate buffer solution of pH 9.2; the colour of the eluate was proportional to the amount of insulin present. We therefore investigated this method and found that we obtained

satisfactory results for the estimation of insulin after chromatographic separation from protamine. A number of other dyes were examined, but none gave better results than bromocresol green.

EXPERIMENTAL

Separation of insulin from protamine by chromatography

In preliminary experiments 10 μ l. drops of a 1% (w/v) protein solution, as well as a mixture of protamine sulphate and insulin solutions, were placed on Whatman no. 1 filter-paper strips (2 cm. broad) and developed with a large number of solvent mixtures. Each of the protein solutions when examined separately gave single spots. The protamine sulphate was derived from salmon milt and the insulin from ox pancreas.

The mixture found to be most satisfactory for the separation of protamine from insulin was the upper phase obtained by equilibrating *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). This solvent mixture gave a clean separation of protamine and insulin with R_F values of 0.00 and 0.43-0.45 respectively.

Retention analysis of proteins

Preliminary experiments were carried out with solutions of insulin, protamine sulphate, blood albumin and edestin, varying the concentrations and volumes of the solutions used. A drop of each solution was placed 2 cm. from the lower edge of a rectangular piece of Whatman no. 1 filter paper, which was suspended vertically in a covered beaker so that the lower edge of the paper dipped about 1 cm. into the dye solution. The dye was allowed to rise about 5 cm. above the protein spots, and the paper was then dried. The white portion above the spot was traced on to graph paper and its area measured.

Using a 0.1% (w/v) solution of erythrosine, the results recorded in Table 1 were obtained. Fig. 1 illustrates the

Table 1. *Effect of concentration and drop size on the retention analysis of protein solutions with erythrosine*

(For experimental procedure see text.)

Protein	Concentration of solution (% w/v)	Volume of drop (μ l.)	Retention area (sq.mm.)	Relative retention areas
Insulin	1	5	61	1.0
	1	10	102	1.7
	1	20	202	3.3
Protamine sulphate	1	5	99	1.0
	1	10	198	2.0
	1	20	356	3.6
Protamine sulphate	0.2	5	81	1.0
	0.4	5	180	2.2
	0.6	5	285	3.5
	0.8	5	365	4.5
Blood albumin	0.125	10	55	1.0
	0.25	10	114	2.1
	0.5	10	167	3.0
Edestin	1	10	18	1.0
	2	10	38	2.1

appearance after retention analysis of filter papers to which 5 μ l. spots of 0.2, 0.4, 0.6 and 0.8% (w/v) protamine sulphate solutions had been applied.

solution; from the positions of the two coloured zones thus formed, the positions of the insulin and protamine on the undyed strip were noted.

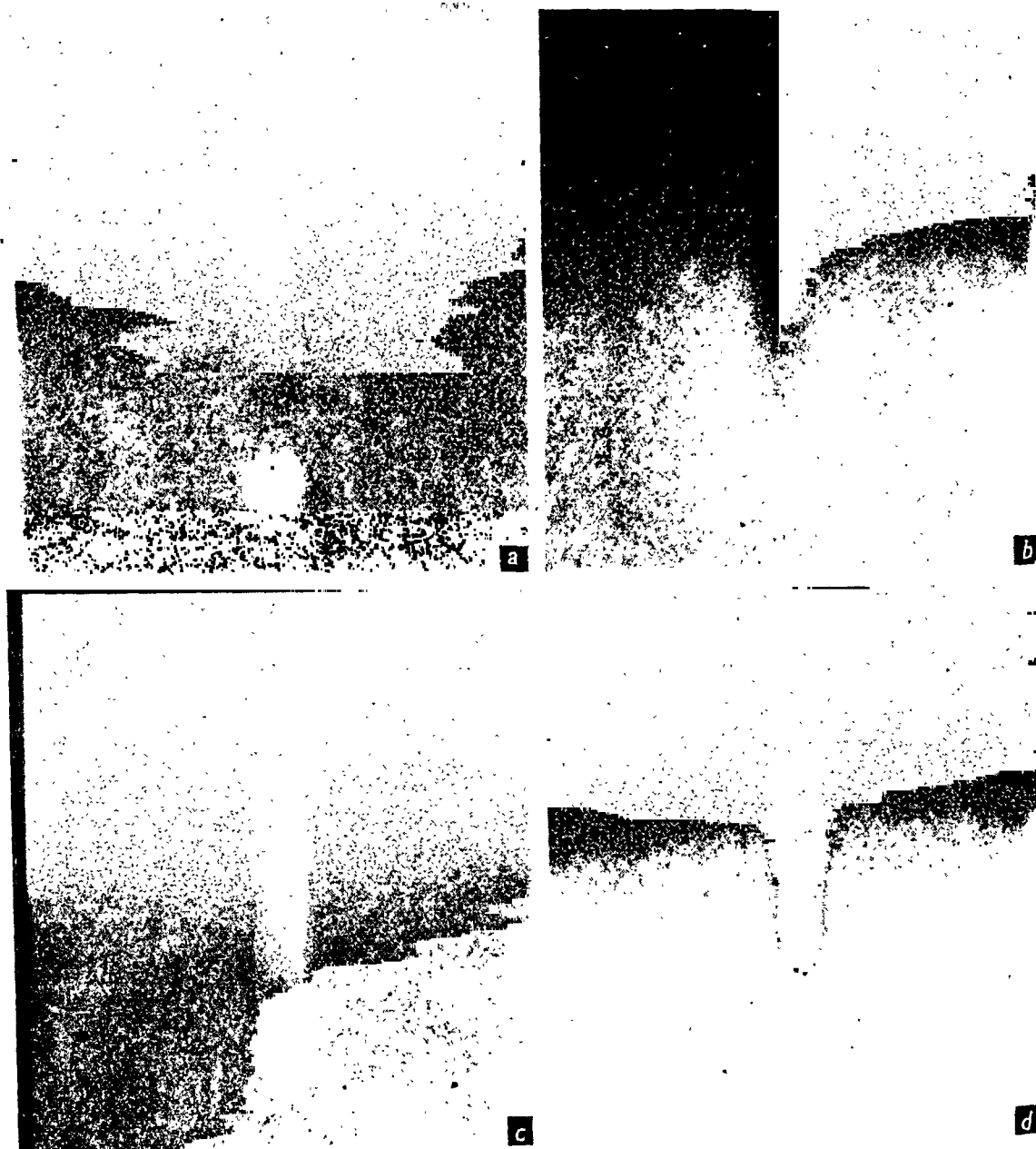


Fig. 1. Paper chromatograms obtained by retention analysis of 5 μ l. spots of (a) 0.2, (b) 0.4, (c) 0.6 and (d) 0.8% (w/v) protamine sulphate solution with 0.1% erythrosine solution.

Attempted estimation of insulin and protamine by retention analysis

Portions (20 μ l.) of 1% insulin solution and of 1% protamine sulphate solution were mixed and half of the mixture applied to each of two 10 cm. strips of Whatman no. 1 filter paper, which were then developed with the aqueous butanol-acetic acid mixture. The strips were dried and one was dipped into a 0.1% (w/v) Solway purple

This assay strip was cut into four portions each about 8 cm. wide, one portion containing the protamine zone, another the insulin zone and the third and fourth portions containing no protein. Measured amounts (10 μ l.) of 1% insulin solution and of 1% protamine sulphate solution were applied to the third and fourth portions respectively to serve as standards.

These four portions of the chromatogram were retention-analysed as described above with a 0.1% solution of

erythrosine, this having been found to work better with protamine than did Solway purple.

The following retention areas were obtained: standard (10 μ l. of 1% insulin solution)=123 sq.mm.; test solution (insulin band)=179 sq.mm.; standard (10 μ l. of 1% protamine sulphate solution)=312 sq.mm.; test solution (protamine band)=320 sq.mm.

From these results the insulin zone is apparently equivalent to 14.5 μ l. of a 1% insulin solution, whereas only 10 μ l. of 1% insulin solution had been chromatographed. The protamine zone, on the other hand, gave an area equivalent to 10.2 μ l. of 1% protamine sulphate solution, a result in excellent agreement with the theoretical value of 10 μ l.

Presumably, therefore, before insulin can be estimated by retention analysis it is essential that a more compact zone should be obtained than is formed when insulin is chromatographed with the solvent mixture described above. In an attempt to obtain a more suitable insulin zone, another chromatogram was prepared as before. The portion containing the protamine zone was cut off and the insulin zones were developed further with dilute acetic acid (1 part

since this remained in the form of a compact spot readily amenable to retention analysis. Various samples of protamine zinc insulin were freed from insulin by paper chromatography and the residual protamine spot was assayed by retention analysis. Portions of the precipitates and supernatant liquors from these samples were also examined in the same way in order to obtain an estimate of the distribution of the protamine between the precipitates and the supernatant solutions. This was done by centrifuging portions of the protamine zinc insulin sample under investigation, separating the supernatant liquor from the precipitate, dissolving the latter in a few drops of dilute HCl and diluting the solution thus obtained to the original volume with distilled water. Portions (50 μ l.) of the original protamine zinc insulin suspension, the supernatant liquor, and the dissolved precipitate were chromatographed and the protamine-containing portions of the filter papers were examined by retention analysis as described above. The protamine content was calculated from the retention areas by reference to a standard curve prepared by treating in a similar manner standard solutions of protamine sulphate. The results are shown in Table 2.

Table 2. *Protamine and insulin contents of various samples of protamine zinc insulin*

(For analytical procedures see text. Figures in table are mg./100 ml. Values given are mean and range. Figures in brackets represent number of assays done.)

Protamine zinc insulin sample no.	Insulin			Protamine		
	Total	Precipitate	Supernatant	Total	Precipitate	Supernatant
AB836 (40 units)	162 \pm 6 (5)	162 \pm 30 (4)	Too low to measure	30 \pm 9 (6)	34 \pm 7 (2)	Too low to measure (2)
C208165 (40 units)	204 \pm 3 (2)	250 (1)	Too low to measure	50 \pm 10 (6)	51 \pm 6 (3)	5 \pm 5 (3)
S919 (40 units)	170 \pm 7 (3)	133 \pm 3 (2)	Too low to measure	36 \pm 10 (6)	39 \pm 6 (3)	4 \pm 4 (4)
SD342442 (40 units)	160 \pm 13 (2)	151 (1)	Too low to measure	41 \pm 11 (5)	31 \pm 10 (2)	Too low to measure (1)
L4134 (40 units)	158 \pm 8 (2)	178 \pm 1 (2)	Too low to measure	42 \pm 8 (5)	46 \pm 3 (2)	3 \pm 3 (2)
O7510 (40 units)	—	—	—	8 \pm 2 (5)	4 \pm 4 (3)	7 \pm 6 (3)
B119 (40 units)	150 \pm 34 (5)	134 \pm 10 (3)	Too low to measure	43 \pm 4 (6)	44 \pm 0 (2)	Too low to measure (1)
B120 (40 units)	155 \pm 18 (7)	156 \pm 1 (2)	Too low to measure	48 \pm 9 (5)	38 \pm 7 (4)	Too low to measure (1)
B121 (40 units)	187 \pm 35 (5)	150 \pm 19 (3)	Too low to measure	51 \pm 6 (6)	42 \pm 1 (2)	Too low to measure (1)
B122 (80 units)	335 \pm 31 (3)	338 \pm 25 (3)	Too low to measure	96 \pm 8 (6)	86 \pm 7 (2)	Too low to measure (2)

glacial acetic acid to 4 parts of water), which had been shown in preliminary experiments to form a compact zone at the solvent front. The resulting zone was retention-analysed as before, with the following results: standard (10 μ l. of 1% insulin solution)=117 sq.mm.; test solution (insulin band)=151 sq.mm. Again the result was higher than anticipated, the area being equivalent to 12.9 μ l. instead of 10 μ l. of 1% insulin solution.

Estimation of protamine in protamine zinc insulin

As the retention analysis of insulin after separation from protamine by chromatography gave unsatisfactory results, attention was concentrated on the estimation of protamine,

Estimation of insulin in protamine zinc insulin

The amount of insulin present in the volume of protamine zinc insulin suspension adequate for the estimation of protamine was found to be insufficient for the estimation of insulin by Dickinson's method. Accordingly, a second chromatographic separation was carried out on a rectangular sheet of Whatman no. 1 filter paper. Up to 400 μ l. of the solution were streaked in a broad band across one end of the paper and about 6 cm. from the edge. The chromatogram was then developed with the upper phase obtained by equilibrating a mixture of *n*-butanol (75%) and acetic acid (25%) with water. After drying, the paper was soaked

in a 0.02% (w/v) aqueous solution of bromocresol green for 5 min. This resulted in adsorption of the dye by both the protamine band and the insulin band. The background colour was removed by washing the strips for 20 min. in three changes of 2% (w/v) acetic acid and the strips were then dried. Fig. 2 illustrates the appearance of a typical chromatogram after exposure to ammonia vapour to intensify the colour. A section 6 cm. wide containing the insulin band was cut out and supported vertically with the upper end bent over and dipping into a borate buffer solution of pH 9.2. This was prepared by mixing 50 ml. of 0.1M-boric acid in 0.2M-KCl solution with 26.7 ml. of 0.2N-NaOH solution and diluting to 200 ml. This eluted the insulin-dye complex, and the eluate, which was collected at the lower end of the strip, was made up to 10 ml.

A 50 μ l. spot of the solution was applied to a rectangular sheet of filter paper (10 \times 30 cm.) 2 cm. from the longer and 8 cm. from the shorter edge. The latter was bent over so that it dipped 3 cm. into the trough of the chromatographic apparatus containing the upper phase of the *n*-butanol-glacial acetic acid-water mixture described above. After development in a closed vessel for about 8 hr., during which time the solvent front travelled the whole length of the paper and the insulin spot moved about 15 cm., the filter paper was dried in a current of warm air. A 3 cm. strip was then cut from the end of the paper that had been immersed in the solvent, and discarded. The remaining sheet was cut into two portions 10 cm. from the new edge thus formed, and the smaller of the two pieces, containing the protamine spot 2 cm. from one edge, was supported with this edge

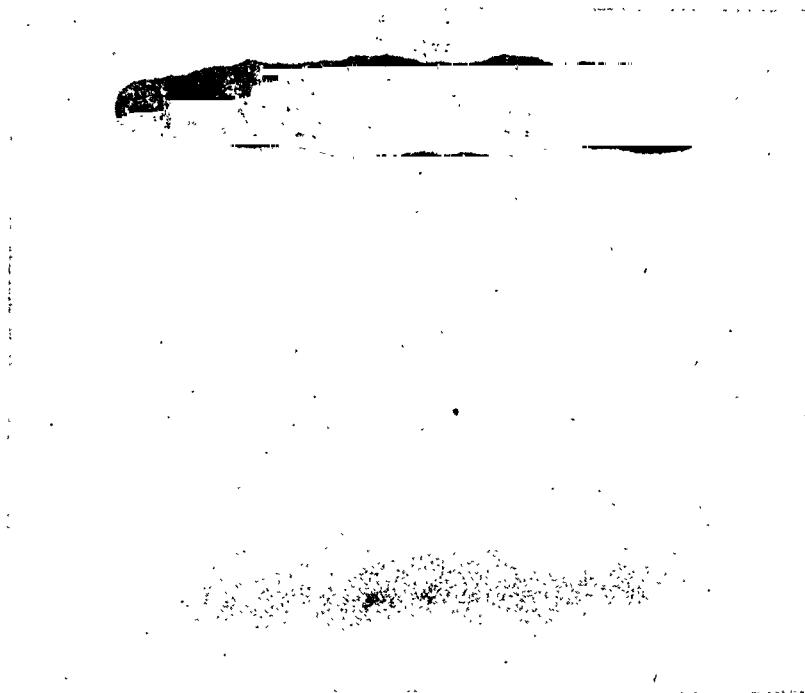


Fig. 2. Paper chromatogram showing bands of protamine (above) and insulin (below) obtained by developing protamine zinc insulin solution with aqueous butanol-acetic acid and staining with bromocresol green solution.

A 6 cm. portion containing no protein band was cut out from the paper and eluted in the same way for use as a blank. The optical density of the eluate was measured against the blank solution in a Spekker absorptiometer using Ilford filter no. 607.

Portions of insulin solutions of various concentrations were chromatographed and the insulin bands treated as described above. A graph was plotted connecting the insulin concentration with the absorptiometer reading. The relation was found to be linear between 0.25 and 1.5% of insulin. The insulin content of the unknown solution was calculated from this standard curve. The results are shown in Table 2.

Recommended method for the estimation of protamine and insulin in protamine zinc insulin

The procedure finally adopted for the estimation of protamine and insulin in protamine zinc insulin suspension was as follows:

dipping into a 0.1% solution of erythrosine and the dye allowed to rise about 5 cm. above the spot. The area of the white V-shaped wedge was then measured by tracing on to graph paper and the results calculated from a standard curve prepared by treating known amounts of protamine in the same manner.

To estimate the insulin content a fresh chromatogram was prepared by streaking 200–400 μ l. of protamine zinc insulin solution about 6 cm. from and parallel to the shorter edge of a rectangular sheet of filter paper similar to that used in the protamine estimation. The chromatogram was developed and dried as before. The paper was dipped into a 0.02% solution of bromocresol green for 5 min. and then washed for 20 min. in three changes of 2% acetic acid to remove the background colour. The paper was again dried, the portion containing the insulin zone was cut out and the insulin-dye complex eluted with a borate buffer solution (pH 9.2) and subjected to absorptiometry exactly as described above.

SUMMARY

1. Insulin can be separated from protamine by paper chromatography developing with the upper phase of a mixture of *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). A dye such as bromocresol green is used for locating the zones.

2. The concentration of protamine and of various proteins in a solution can be estimated on filter paper by retention analysis with a suitable dye, e.g. erythrosine. The area of the unstained wedge formed above the protein spot is proportional to the amount of protein present. The method can be used to estimate the amount of protamine in protamine zinc insulin after removal of insulin by paper chromatography.

3. The concentration of insulin in protamine zinc insulin can be estimated, after separating the insulin from protamine by paper chromatography, by staining with bromocresol green solution, eluting the insulin-dye complex and comparing the colour of the eluate with that given by a standard solution of insulin treated in the same way.

4. Data are recorded on the composition of a number of suspensions of protamine zinc insulin and on the distribution of the components between the phases of the suspension.

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Studies on Cholinesterase

7. DETERMINATION OF THE MOLAR CONCENTRATION OF PSEUDO-CHOLINESTERASE IN SERUM

By D. K. MYERS

Pharmaco-therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland

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The classical theory of reversible enzyme-substrate and enzyme-inhibitor reactions postulates that enzyme and substrate, or enzyme and inhibitor, form a reversible combination according to mass law principles (Michaelis & Menten, 1913). The mechanism of enzyme-inhibitor reactions has been analysed from this theoretical standpoint by Straus & Goldstein (1943) and Goldstein (1944), with special consideration being given to the concentration of free enzyme and free inhibitor. They pointed out that the degree of inhibition of enzyme activity effected by a certain concentration of inhibitor will be dependent in part upon the enzyme concentration when the so-called 'dissociation' or Michaelis constant of the enzyme-inhibitor system is of the same order of magnitude or much smaller than the molar enzyme concentration employed.

The theoretical treatments given by Goldstein (1944) were illustrated with experimental data obtained, using a pseudo-cholinesterase preparation

as the source of enzyme and eserine as the enzyme inhibitor. In this system it is possible to obtain appreciable inhibition of the cholinesterase activity with eserine concentrations as low as 10^{-8} M; nevertheless, the degree of inhibition is practically independent of enzyme concentration under the experimental conditions. Thus it was not possible to confirm the theoretical formulations completely at that time, or to arrive at any definite value for enzyme concentration in the cholinesterase system.

Since that time, other investigators, notably Bain (1949), have attempted to determine cholinesterase concentration by the use of irreversible alkyl-phosphate inhibitors such as diisopropyl fluorophosphonate (DFP) and tetraethyl pyrophosphate (TEPP). The lack of theoretical criteria which could be applied to the results with these inhibitors makes this a rather unreliable method for determining cholinesterase concentration. Moreover, the results obtained do not correspond with those expected of

an irreversible inhibitor which combines specifically and only with the active centre of the cholinesterase on an equimolar basis.

However, it has been observed that the reversible inhibition of pseudo-cholinesterase by the very potent inhibitor, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Nu683), is dependent upon the cholinesterase concentration (Myers, 1950) and that the experimental results obtained correspond with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) for a competitive reversible inhibitor of high potency. Evidently the concentrations of Nu 683 which produce appreciable inhibition of pseudo-cholinesterase activity are of the same order of magnitude as the concentrations of pseudo-cholinesterase. By applying the theoretical formulations given by Goldstein, pseudo-cholinesterase concentration in serum has been calculated by a method which appears to be completely justified theoretically.

METHODS

The activity of the cholinesterases was measured manometrically by a method similar to those described previously (Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1950). The medium used was a 0.025M-bicarbonate solution saturated with 5% CO₂ + 95% N₂ (v/v), pH 7.4 at 37.5°. Dialysed human, horse, dog or rat serum was used as source of pseudo-cholinesterase, usually with 0.006M-benzoylcholine as substrate (Mendel, Mundell & Rudney, 1943). Esterase activity for a wide range of cholinesterase concentrations was kept within accurately measurable range by varying the total volume of solution between 2.0 and 5.0 ml. and by the use of 0.06M-acetylcholine at very low enzyme concentrations with horse and human serum. The cholinesterase activity was, however, always calculated out to the value it would have had if 5.0 ml. of the same solution had been used and if benzoylcholine had been the substrate.

The pseudo-cholinesterase inhibitor in most of these experiments was Nu683, described by Hawkins & Gunter (1946). This inhibitor is still not sufficiently potent to allow the calculation of enzyme concentration from inhibition in a system where enzyme, inhibitor and substrate are all equilibrated, as has been recommended by Goldstein (1944). Therefore, the measurements must of necessity be done with a system in which only enzyme and inhibitor are in equilibrium. For a competitive reversible enzyme-inhibitor system of this kind, the relationship between inhibitor concentration I and fractional activity a is expressed as

$$I = K_I \frac{(1-a)}{a} + (1-a)E \quad (\text{Goldstein, 1944}).$$

The concentration of inhibitor required to produce 50% inhibition of the enzyme activity can be designated as I_{50} . When the ratio $E/K_I < 0.1$, the concentration I_{50} will be approximately equal to K_I and independent of the enzyme concentration E (zone A). When $E/K_I > 100$, the concentration I_{50} will be directly proportional to E and independent of the Michaelis constant K_I (zone C). For intermediate values of E/K_I , I_{50} will depend upon the values of both

E and K_I (zone B) (Straus & Goldstein, 1943; Goldstein, 1944). As will be shown below, the pseudo-cholinesterase-esterine system used by Goldstein (1944) must have been in zone A. The pseudo-cholinesterase-Nu683 system used in the present investigation usually lies in the lower part of zone B so that the values of both E and K_I can be accurately determined.

The equation above can be applied only when sufficient time has been allowed to ensure complete equilibration of the enzyme-inhibitor system. The time of incubation should not be too long, however, or errors may be introduced by the slow enzymic hydrolysis of the carbamic acid ester linkage in the combined inhibitor. Another factor is the progressive displacement of combined inhibitor and consequent increase in the fractional activity caused by the addition of substrate to the equilibrated enzyme-inhibitor system. The importance of these three factors has been stressed by Goldstein (1944), but, as will be described below, the possible errors which they would introduce are relatively small in the case of the enzyme-inhibitor system with Nu683 and the pseudo-cholinesterase of human or horse serum. During the course of these investigations, preliminary experiments were done with the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu1250) (Hawkins & Mendel, 1949) as inhibitor of the true cholinesterase activity of purified human erythrocytes (Mendel & Rudney, 1943). In this case the rate of displacement of combined inhibitor by added substrate proved to be appreciable under the experimental conditions. In addition, the ratio E/K_I was still quite low so that the shift in I_{50} was relatively small. Both of these factors increase the possibility of error in the estimations of enzyme concentration and consequently this enzyme-inhibitor system did not seem suitable for an accurate determination of cholinesterase concentration.

For purposes of greater accuracy with Nu683, the value of I_{50} at each enzyme concentration was estimated from a series of determinations with different inhibitor concentrations covering the range from 10 to 90% inhibition of the pseudo-cholinesterase activity. The precise value of I_{50} was obtained by plotting the fractional activity a against pI ($-\log I$) for each set of results, as recommended by Goldstein (1944). The theoretical equation relating inhibitor to enzyme concentration then becomes $I_{50} = K_I + 0.5E$. If I_{50} is determined for a series of different enzyme concentrations, a plot of I_{50} against the relative concentration of enzyme should give a straight line with intercept equal to K_I and a slope equal to one-half the molar concentration of enzyme.

RESULTS

The most convenient system for accurate determination of pseudo-cholinesterase concentration was that with Nu683 as inhibitor and the pseudo-cholinesterase of dialysed human serum as enzyme. It had already been shown (Myers, 1950) that this enzyme-inhibitor system exhibits the predicted characteristics (Goldstein, 1944) of a competitive reversible system for which the ratio of E/K_I has a value appreciably greater than 0.1 and in which the inhibitor combines specifically with the enzyme active centre on an equimolar basis ($n=1$). This is further illustrated by Fig. 1, in which typical

experimental points are compared with the theoretical curves expected for an enzyme-inhibitor system in zone B ($0.1 < E/K_I < 100$) (see a later section for theoretical treatment).

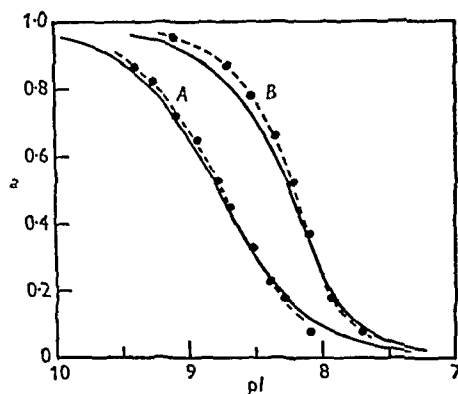


Fig. 1. Comparison of the theoretical curves (—) expected for a competitive reversible inhibitor in zone B with the experimental curves (-----) obtained using Nu683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum. Theoretical curves calculated for $K_I = 8.8 \times 10^{-10}$ M-Nu683 and $E = 2.3 \times 10^{-9}$ M-pseudo-cholinesterase/100 μ l. CO_2 activity. Experimental points of curve A obtained after 50 min. incubation of the Nu683 with a 2% (v/v) serum solution giving 70 μ l. CO_2 /5 ml. solution/20 min.; those of curve B with a 12% (v/v) serum solution giving 448 μ l. CO_2 /5 ml. solution/20 min. pI represents the negative log. of the molar concentration of Nu683 and a the fractional activity in the presence of this inhibitor.

Table 1. Effect of added benzoylcholine in displacing Nu683 combined with the pseudo-cholinesterase of human serum

(5 ml. of 6% (v/v) dialysed human serum in 0.025 M-bicarbonate was incubated with 6×10^{-9} M-Nu683 for 50 min. at 37.5° before adding 0.006 M-benzoylcholine at time = 0.)

Time after addition of benzoylcholine (min.)	Activity, for time stated, of control serum (μ l. CO_2)	Activity, for time stated, of serum incubated with Nu683 (μ l. CO_2)	Fractional inhibition by Nu683 ($1 - a$)
2-7	49.5	12.0	0.758
7-12	55.5	13.0	0.766
12-17	52.5	14.5	0.724
17-22	55.5	15.0	0.730
Average 2-22 min.			0.745
22-32	112.0	32.5	0.710
32-42	114.5	36.5	0.681
42-52	110.5	38.5	0.651
52-62	112.0	43.0	0.616
62-72	111.5	47.5	0.574
72-82	112.5	50.0	0.556
82-92	114.0	55.0	0.517

The error introduced by the displacement of combined inhibitor after addition of substrate was

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relatively small when the activity was measured over the period 2-22 min. after the addition of benzoylcholine (Table 1). However, the fact that Nu683 combined with pseudo-cholinesterase was displaced by added benzoylcholine does indicate that Nu683 is a reversible competitive inhibitor of pseudo-cholinesterase.

It was also necessary, as noted previously, to determine an optimal time of incubation of the enzyme with inhibitor, that is, a time which would be long enough to ensure relatively complete equilibration of the enzyme-inhibitor, but not so long as to allow any appreciable destruction of inhibitor to take place. To this end, a series of determinations of pI_{50} ($-\log I_{50}$) after various times of incubation was carried out using 2 and 12% serum concentrations. The results obtained are given in Table 2; from this data, a standard time of 50 min. incubation was arbitrarily selected for use in subsequent determinations.

Table 2. Changes in pI_{50} after incubation of 2 and 12% (v/v) solutions of human serum with Nu683 for various times at 37.5°

Time of incubation (min.)	pI_{50}	
	2% serum	12% serum
30	8.83	8.18
50	8.77	8.21
75	8.79	8.07
160	8.64	7.92
270	8.53	7.62

A series of experimental a -pI curves, similar to those shown in Fig. 1, was determined for a total of fifteen different enzyme concentrations with several samples of serum. From each of these curves a value of I_{50} was calculated. The enzyme activity was directly proportional to the serum concentration with any one sample of serum (Fig. 2), but the activity per unit volume varied from one sample to another, as would be expected. Therefore the relative enzyme concentration was expressed on the basis of enzyme activity. A plot of I_{50} against relative enzyme concentration gave the experimental points of Fig. 3. The best straight line, fitted to these points by the method of least squares, proved to be that represented by the equation $I_{50} \times 10^9 = 0.880 + 1.15$ (per 100 μ l. activity).

From the theoretical equation $I_{50} = K_I + 0.5E$, it follows that $K_I = 8.8 \times 10^{-10}$ M-Nu683 and

$$E = 2.3 \times 10^{-9} \text{ M-pseudo-cholinesterase}$$

in a solution of which 5 ml. gave an activity of 100 μ l. CO_2 /20 min. with 0.006 M-benzoylcholine as substrate, when measured under the standard conditions of 37.5° , pH 7.4 and 0.025 M-sodium bicarbonate medium. This same concentration of the pseudo-cholinesterase of human serum would

give an activity of 292 $\mu\text{l. CO}_2/20 \text{ min.}$ under the same conditions when using 0.06M-acetylcholine as substrate.

An enzyme activity of this order of magnitude is usually obtained with a concentration of about 3% human serum. For a full serum which gives an activity of 700 $\mu\text{l. CO}_2/\text{ml. serum}/20 \text{ min.}$ with benzoylcholine, the concentration of pseudo-cholinesterase would be precisely 35 times as high as the figure given above. That is to say, the concentration of pseudo-cholinesterase in full human serum would be about $8 \times 10^{-8} \text{M}$ on the average.

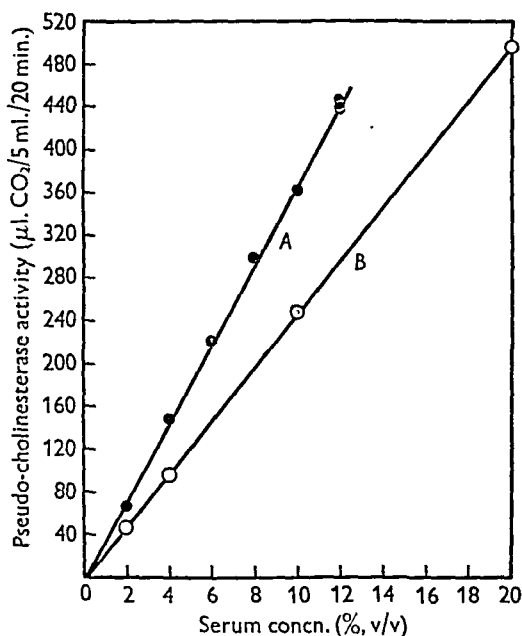


Fig. 2. Linear relationship between the pseudo-cholinesterase activity and the concentration of serum used for two different samples of dialysed human serum. The original activity of the full serum corresponding to line A was 820 $\mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$; this was diluted to 1.12 times the original volume in the process of dialysis. The full serum corresponding to the dialysed sample of line B had an activity of 685 $\mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$ and was diluted to 1.38 times the original volume in the process of dialysis. The activity was determined using 0.006M-benzoylcholine as substrate, a fluid volume of 5 ml. below 10% (v/v) dialysed serum and a fluid volume of 2.5 ml. above the 10% serum concentration, a rate of shaking of $150 \times 5 \text{ cm. strokes/min.}$, and the other conditions as described in the method.

A similar type of determination on dialysed dog serum gave an equation (from 5 points) $I_{50} \times 10^3 = 3.2 + 1.35$ (per 100 $\mu\text{l.}$ activity), from which $K_i = 3.2 \times 10^{-9} \text{M-Nu 683}$ and $E = 2.7 \times 10^{-9} \text{M-pseudo-cholinesterase}$ in a solution of which 5 ml. gave an activity of 100 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.006M-benzoylcholine under the standard experimental conditions. In this case the ratio E/K_i would be approximately 0.8 for the pseudo-cholinesterase

concentration in a solution which gives the particular esterase activity selected as standard, whereas in the case of human serum previously the two values obtained would give a ratio $E/K_i = 2.6$. From the theoretical treatments of Straus & Goldstein (1943) and Goldstein (1944), it would be expected that the enzyme-inhibitor system is much

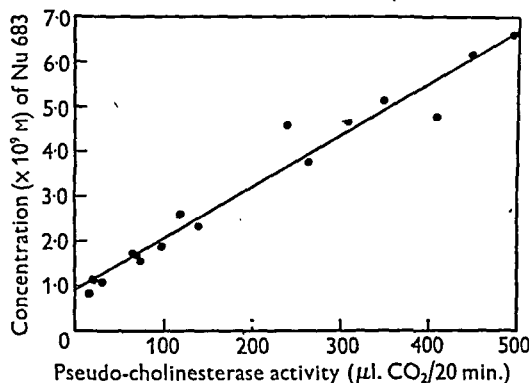


Fig. 3. Relationship between the relative concentration of pseudo-cholinesterase and the molar concentration of Nu 683 which would cause 50% inhibition of the pseudo-cholinesterase activity of dialysed human serum towards 0.006M-benzoylcholine.

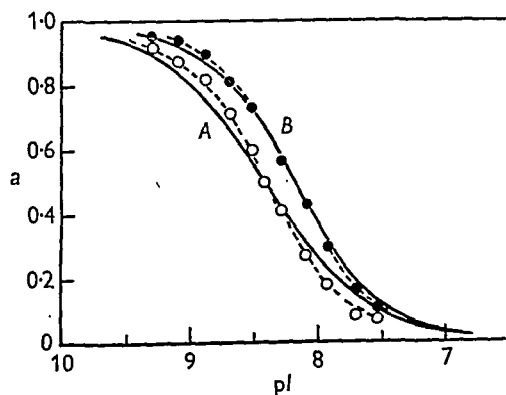


Fig. 4. Comparison of the theoretical (—) and experimental (-----) curves obtained using Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed dog serum. (cf. Fig. 1). Experimental points of curve A obtained after 50 min. incubation of the Nu 683 with a 2% (v/v) serum solution giving 35.4 $\mu\text{l. CO}_2/5 \text{ ml. solution}/20 \text{ min.}$; those of curve B with a 12% (v/v) serum solution giving 206 $\mu\text{l. CO}_2/5 \text{ ml. solution}/20 \text{ min.}$

closer to zone A with dog serum than with human serum under the conditions used. In agreement with this fact, the a - pI curves obtained were somewhat flatter and less widely spread than those obtained with human serum. Also the experimental points agree relatively well with the theoretical curves expected from the values of E and K_i obtained (Fig. 4). It is a striking fact that these two sets of curves (Figs. 1 and 4) give similar values

for the pseudo-cholinesterase concentration per unit of activity despite their differences in shape and relative position.

The same concentration of dog serum gave an activity of 186 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.06M-acetylcholine as substrate. From this it can be calculated that the concentration of pseudo-cholinesterase in the 4.54% dog serum used by Goldstein (63.1 $\mu\text{l. CO}_2/20 \text{ min.}$ with a volume of 2.2 ml. and with 0.08M-acetylcholine as substrate) was probably $2.07 \times 10^{-8}\text{M}$, as compared with the value $E < 1.8 \times 10^{-8}\text{M}$ used by Goldstein (1944). This would mean that the ratio E/K_i with eserine as inhibitor ($K_i = 3.11 \times 10^{-8}\text{M}$) was of the order of 0.067 in Goldstein's investigations, and justifies the application of zone A equations to the experimental data obtained by Goldstein (1944) with eserine as the pseudo-cholinesterase inhibitor.

pseudo-cholinesterase in the serum. In human serum or horse serum, however, the amounts of true cholinesterase present are so small that no appreciable error will be introduced by the use of acetylcholine as substrate for the pseudo-cholinesterase.

Similar determinations of the values of E and K_i were also carried out with the pseudo-cholinesterases of horse, rat and mouse sera. The relative activities of the pseudo-cholinesterases of rat and mouse serum towards acetylcholine were determined by the use of Nu1250 and half-saturated ammonium sulphate as previously described for dog serum. The results obtained are summarized in Table 3.

Difficulties were encountered with rat serum in that the relatively low pseudo-cholinesterase activity of the full serum does not permit such a wide range of enzyme concentrations to be used in

Table 3. Comparison of the values of K_i and E as determined with Nu683 for the pseudo-cholinesterases from the sera of different species

Source of serum	Activity of full serum towards benzoylcholine ($\mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$)	No. of determinations of I_{50}	K_i ($\times 10^{-8}\text{M}$) Nu683	E_{BCh}^* ($\times 10^{-9}\text{M}$)	ACh/BCh†	E_{ACh}^* ($\times 10^{-10}\text{M}$)
Mouse	355	7	0.56	3.27	8.2	3.98
Rat	180	8	3.3	2.05	4.25	4.82
Horse	790	7	0.72	2.24	3.35	6.70
Man	685-820	15	0.88	2.30	2.92	7.89
Dog	340	5	3.2	2.70	1.62	16.7

* E_{BCh} represents the molar concentration of pseudo-cholinesterase in a solution of which 5 ml. give an activity of 100 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.006M-benzoylcholine as substrate under the standard experimental conditions used. E_{ACh} defined similarly but with 0.06M-acetylcholine as substrate.

† ACh/BCh is the ratio of pseudo-cholinesterase activity towards 0.06M-acetylcholine over that towards 0.006M-benzoylcholine.

However, it should be noted that dog serum usually contains appreciable amounts of true cholinesterase as well as the pseudo-cholinesterase, and that both of these enzymes are capable of hydrolysing acetylcholine even at high concentrations of this substrate (Mendel & Rudney, 1943; Mendel *et al.* 1943).

To determine the relative pseudo-cholinesterase activity towards acetylcholine and benzoylcholine, it was first necessary to eliminate all true cholinesterase activity towards acetylcholine. This was done by two methods, by selectively inhibiting the true cholinesterase activity with 10^{-7}M -Nu1250 (Hawkins & Mendel, 1949), and by precipitating the true cholinesterase from the serum by addition of an equal volume of saturated ammonium sulphate to the serum (Hawkins & Mendel, 1949). The residual pseudo-cholinesterase activity towards acetylcholine and benzoylcholine was subsequently determined in both cases. With the above example of dog serum, it appeared that only 162 of the 186 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.06M-acetylcholine as substrate were actually due to the activity of the

these determinations as in the other cases. It is known that female rat serum contains much larger amounts of pseudo-cholinesterase than the serum of male rats (Mundell, 1944); the same is also true to a lesser extent for the sera of male and female mice. Therefore, pooled samples of female rat serum and of female mouse serum were employed for these determinations. The pseudo-cholinesterase activity of the female rat serum was 180 $\mu\text{l. CO}_2/\text{ml. serum}/20 \text{ min.}$ with benzoylcholine as substrate; this is relatively high for rat serum, but even so the activity is still somewhat lower than that of the other types of sera used. In addition, the ratio E/K_i was relatively low so that the values of E and K_i could not be determined as accurately as with human serum or horse serum.

DISCUSSION

The absolute activity of pseudo-cholinesterases

The results given in Table 3 show that the pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-

cholinesterase activity towards 0.06M-acetylcholine over that towards 0.006M-benzoylcholine becomes progressively smaller. The values range from 8.2 to 1.6, a difference of 5.1-fold. In this same series, the pseudo-cholinesterase concentration which gives one unit of activity towards acetylcholine becomes progressively larger, that is to say, the absolute activities of the pseudo-cholinesterases in this series becomes progressively smaller. The values obtained cover a range of 4.2-fold. This is a surprising finding, but closer examination of the results show that there is not a close correlation between the figures in these two series. Moreover, the absolute activity of the pseudo-cholinesterases towards benzoylcholine do not form a parallel series. Thus it seems doubtful that there might be some theoretical significance for the rough correlation in this series of pseudo-cholinesterases between the absolute activity towards acetylcholine and the ratio of activity towards acetylcholine over that towards benzoylcholine.

The Michaelis constant for Nu683 also varies considerably from one pseudo-cholinesterase to the other, but varies independently of the other characteristics determined. Measurements of the Michaelis constant for acetylcholine show a similar lack of correlation with any of the other results.

From Table 3 it is apparent that the absolute activity of the pseudo-cholinesterases towards benzoylcholine does not vary as much from one type of serum to another as does the absolute activity towards acetylcholine. The former varies by only 1.50 times for the five preparations of pseudo-cholinesterase investigated, the latter by 4.20 times.

The absolute activity, however, is inversely proportional to the pseudo-cholinesterase concentration which gives one unit of activity, regardless of the particular units used. Easson & Stedman (1936) have defined the term absolute activity in terms of a turnover number, i.e. the number of molecules of substrate hydrolysed by one molecule of pseudo-cholinesterase per unit of time. Unfortunately the values given by these authors are not strictly comparable with the above. However, for purposes of comparison with results on other enzymes, turnover numbers can be calculated from the above results on the basis that

100 μ l. CO_2 /5 ml. soln./20 min.

= 1 μ l. CO_2 /ml. soln./min.

= 1 ml. CO_2 /l. soln./min.

= 4.46×10^{-5} moles/l. soln./min.

If this latter figure is divided by any one of the enzyme concentrations given in Table 3 the corresponding turnover number will be obtained, expressed in terms of min.^{-1} . The enzyme concentrations are expressed simply in moles of enzyme per litre, but inherent in this method of determining enzyme concentration is the assumption that these

concentrations are equivalent to the molar concentration of enzyme active centres. For the present it can only be assumed, in most cases, that each active centre corresponds to one molecule of the enzyme protein.

Thus, for example, each active centre of the pseudo-cholinesterase of horse serum will hydrolyse 6.67×10^4 mol. of acetylcholine or 1.99×10^4 mol. of benzoylcholine per min. under the experimental conditions used. (Compare the value 9.35×10^4 mol. of acetylcholine per min. reported by Berry (1951) after the completion of this paper.) With propionylcholine or butyrylcholine as substrate, this value would be considerably higher. As judged from the results of Augustinsson & Nachmansohn (1949) and Sturge & Whittaker (1950), this turnover number might approach a value of 1.4×10^5 mol. of butyrylcholine per min. A turnover number expressed in this way will be identical with the destruction constant k_d of Goldstein (1944) when the appropriate small correction is made for the effect of substrate concentration.

Another point of some interest is the order of magnitude of the activity of pure pseudo-cholinesterase. The exact molecular weight of pseudo-cholinesterase is unknown, but for a serum albumin we might assume a molecular weight of 70 000 (Kekwick, 1938). Further, assuming that one active centre corresponds to 1 mol. of pseudo-cholinesterase protein, it could be calculated that pure pseudo-cholinesterase from horse serum would have a $Q_{\text{acetylcholine}}$ value of 1 300 000 μ l. CO_2 /mg. dry wt./hr. This is of a similar order of magnitude to the $Q_{\text{acetylcholine}}$ values of 400 000 and 600 000 for the purest preparations of pseudo-cholinesterase obtained by Strelitz (1944) and Mendel & Mundell (1943) respectively.

The data presented in Table 3 show that the pseudo-cholinesterases from the sera of different species of animal may have considerably different affinities for the same inhibitor. Moreover, the absolute rate of hydrolysis (per mole of enzyme) of the same substrate may also differ from one preparation of pseudo-cholinesterase to another. The magnitude of these variations in the absolute rate of hydrolysis by various pseudo-cholinesterase preparations also depends upon the substrate concerned. This fact has already been indicated by the results of other investigators, which show that the pseudo-cholinesterase from horse serum differs from the pseudo-cholinesterase of human serum in the relative rate of hydrolysis of non-choline esters (Blaschko & Holton, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950).

It has been pointed out by Mendel and co-workers (Mendel & Rudney, 1943; Mendel *et al.* 1943; Mendel & Mundell, 1943; Strelitz, 1944; Hawkins & Gunter, 1946; Hawkins & Mendel, 1949) that pseudo-

cholinesterase is a distinct enzyme, with the same general and characteristic specificity towards certain substrates and certain inhibitors in all cases investigated, regardless of the source of the enzyme. The results of later investigations emphasize the fact that preparations of pseudo-cholinesterase from different sources often show minor differences in the relative specificity for various substrates and inhibitors. Consequently, it has been proposed that the pseudo-cholinesterases from various sources should be regarded as a special group or 'family' of related enzymes rather than as a special enzyme, and that the true cholinesterases, similarly, should be regarded as another special group of closely related enzymes (Bodansky, 1946; Augustinsson, 1948). The results of the present investigation would appear to justify such a concept—to the same extent as it would be justified to consider the haemoglobins, for example, as a group of closely related haem proteins.

In both cases it can be considered that the active centre or active grouping will determine the general characteristic specificity common to all different preparations of the same enzyme, while the minor variations in relative specificity will be determined mainly by the influence of the various species-specific (and possibly organ-specific) protein carriers. Thus it will depend upon the purposes of the investigation in hand whether the pseudo-cholinesterase (or the true cholinesterase, or the haemoglobin) from different sources is to be regarded as a special enzyme (from the viewpoint of physiological function) or as a special group of enzymes (from the viewpoint of enzymic or immunological investigations). The pseudo-cholinesterases from different sources would appear to have the same general characteristics in all cases, but it must be emphasized that the Michaelis constants, reaction velocity constants and enzyme concentration will not be identical from one preparation of pseudo-cholinesterase to another.

*The use of inhibitors in the study of
pseudo-cholinesterases*

It has been found previously that the inhibition of pseudo-cholinesterase by Nu 683 is reversed only very slowly by dialysis or dilution of the enzyme-inhibitor system (Hawkins & Gunter, 1946). Nevertheless, Nu 683 must be a reversible competitive inhibitor of pseudo-cholinesterase since the combined inhibitor is displaced by added substrate. The previous findings are probably to be explained by the very high affinity of Nu 683 for pseudo-cholinesterase and by the fact that the enzyme-inhibitor system lies in zone B under the experimental conditions.

The experimental results with this inhibitor show sufficiently good agreement with the theoretical

predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the theoretical treatments of these authors, in so far as the concept of three zones of inhibitor action is concerned. And conversely, it would also indicate that Nu 683 combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. Thus the results with this inhibitor should give an accurate determination of pseudo-cholinesterase concentration with the mathematical methods used.

On the other hand, the use of irreversible inhibitors such as DFP or TEPP can lead to erroneous values for enzyme concentration. In the first place it might be noted that the data of Bain (1949) indicate apparent enzyme concentrations which differ by 250 times when these two compounds were used as 'specific' irreversible inhibitors of the true cholinesterase activity of rat brain. Evidently, at least one of the two cannot be a specific cholinesterase inhibitor, but there is no theoretical criterion which could be applied to such results to indicate when an irreversible inhibitor does combine specifically with the cholinesterase active centre on an equimolar basis.

The discrepancies observed in the results with these alkyl-phosphate inhibitors are further illustrated by the data obtained using DFP as an inhibitor of true cholinesterase activity (Fig. 5). The results obtained in this case are more typical of a reversible competitive inhibitor in zone B than of a specific irreversible inhibitor, despite the fact that for most practical purposes the inhibition of cholinesterase activity by DFP is completely irreversible under these experimental conditions. But while the experimental results with DFP would indicate an apparent enzyme concentration of 2.9×10^{-8} moles/100 μ l. activity with 0.03M-acetyl- β -methylcholine under standard conditions, the results obtained with Nu 1250 showed that the true enzyme concentration was no greater than 6.3×10^{-10} M in the same solution. The reactions of cholinesterases with DFP and similar inhibitors will be dealt with in more detail in a subsequent publication. For the present purposes it is sufficient to note that the explanation of these results with DFP is still uncertain, and that the use of this inhibitor will not give a reliable value for the concentration of cholinesterase under the above conditions.

At least three other groups of investigators (Easson & Stedman, 1936; Ellis, Plachte & Straus, 1943; Straus & Goldstein, 1943) have attempted to measure the pseudo-cholinesterase concentration in horse serum by the use of eserine or prostigmine as inhibitors. Their results have been criticized on theoretical and practical grounds by Goldstein (1944), who himself did not attempt to give anything more than a maximum value for the cholinesterase concentration. The results obtained in the present

investigation with Nu 683 as inhibitor do indicate considerably different concentrations of pseudo-cholinesterase in horse serum from those which have been previously proposed, as would be expected in view of Goldstein's criticism.

The difference between the method used in the present investigation for determination of pseudo-cholinesterase concentration and previous determinations with competitive reversible inhibitors

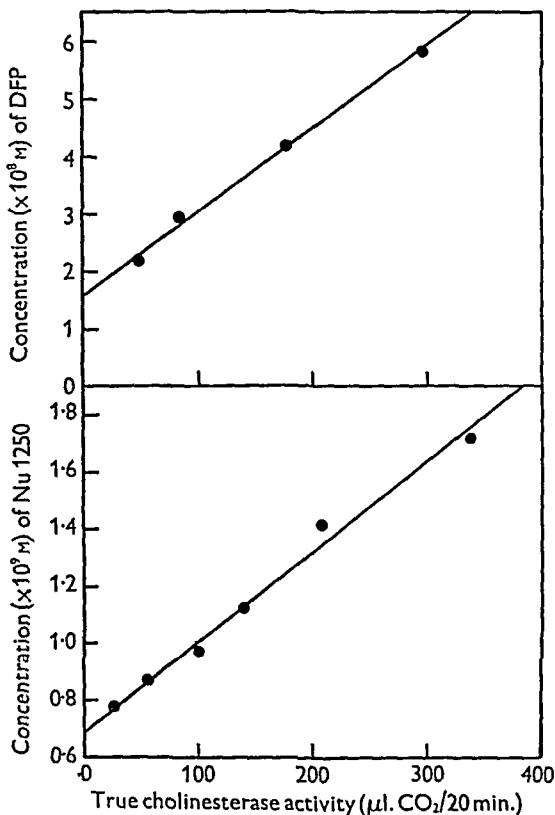


Fig. 5. Relationship between the relative concentration of true cholinesterase and the molar concentrations of Nu 1250 or of DFP which would cause 50% inhibition of the true cholinesterase activity of human erythrocytes towards 0.03M-acetyl-β-methylcholine. The degree of inhibition effected by various concentrations of Nu 1250 was determined after incubation with the erythrocyte preparation for 80 min., that effected by DFP after incubation for 120 min.

lie in three major points. In the first place, Nu 683 is a much more potent inhibitor of pseudo-cholinesterase than either eserine or prostigmine; consequently the degree of inhibition obtained is more dependent on the enzyme concentration. Secondly, the enzyme concentration and Michaelis constant are determined from the results at several different enzyme concentrations rather than from one set of results as in the generally accepted method of Easson & Stedman (1936). Thirdly, the experimental results are plotted as fractional activity a

against pI to give a sigmoid curve. Easson & Stedman (1936), on the other hand, used an equation of the form

$$\frac{I}{(1-a)} = \frac{K_I}{a} + E,$$

and plotted $I/(1-a)$ against $1/a$ on a linear basis. The equation itself is theoretically correct, of course, and this mathematical method of analysing the results would give correct values for E and K_I if the experimental results correspond exactly with the theoretical expectations. But when the data are subject to some experimental error, the use of this equation can give results which are very misleading. Goldstein (1944) has pointed out that this latter method gives a vastly disproportionate weight to the experimental results with very small values of a , and that it is just these results more than any others which are most subject to large experimental errors. The method of plotting a against pI would weight all points to the same extent so that a theoretical curve can be obtained which will give a minimal standard deviation from all of the experimental points (Goldstein, 1944). Thus this method will give a more accurate representation of the experimental data, and the shift in I_{50} concentration with changes in enzyme concentration will allow the calculation of a reliable value for the molar concentration of enzyme. The a - pI plot has a further advantage in that the experimental results can be compared with the theoretical predictions for a competitive reversible inhibitor which combines specifically with the enzyme active centre on an equimolar basis. The combination of all of these factors seems to make this a reliable method for the accurate determination of pseudo-cholinesterase concentration in serum.

SUMMARY

1. The experimental results show that Nu 683 (the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide) is a reversible competitive inhibitor of pseudo-cholinesterase which combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. The ratio of E/K_I has a value appreciably greater than 0.1; this has made it possible to determine pseudo-cholinesterase concentration in serum accurately by a method which appears to be completely justified theoretically. The advantages of this method over those which have been used previously have been discussed.

2. The experimental results obtained with this inhibitor show sufficiently good agreement with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the concept of three zones of inhibitor action.

3. The pseudo-cholinesterase concentrations have been expressed on the basis of enzyme activity under standard conditions. The concentration of pseudo-cholinesterase in the particular samples of full serum used were as follows: horse serum 8.8×10^{-8} M; human serum, 7.9×10^{-8} M; female mouse serum, 5.8×10^{-8} M; dog serum, 4.6×10^{-8} M; female rat serum, 1.7×10^{-8} M.

4. The pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-cholinesterase activity towards 0.06 M-acetylcholine over that towards 0.006 M-benzoylcholine diminishes progressively from 8.2 to 1.6. In this same series, the absolute activity of the pseudo-cholinesterases towards acetylcholine becomes progressively smaller. Other characteristics

of the pseudo-cholinesterases also vary considerably from one type of pseudo-cholinesterase to another, but do not show any correlation to the above series.

5. The significance of these results has been discussed with respect to the concept of the pseudo-cholinesterases as a special group of closely related enzymes.

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The Chemical Estimation of Adrenaline-like Substances in Blood

By H. WEIL-MALHERBE AND A. D. BONE

Research Department, Runwell Hospital, Wickford, Essex

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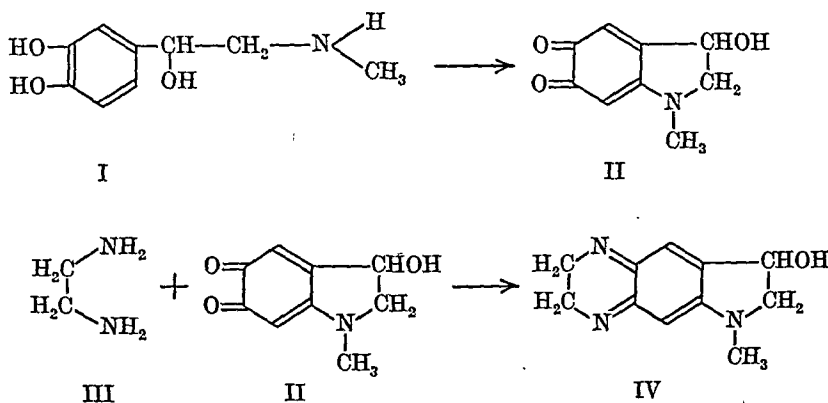
Of the numerous chemical methods which have been devised for the estimation of adrenaline the fluorimetric techniques seem most likely to reach a degree of sensitivity sufficient for the measurement of concentrations as low as those occurring in peripheral venous blood. Adrenaline, as is well known, forms an unstable fluorescent oxidation product in alkaline solution. Lund (1949*b*) recently described a method in which the fluorescent derivative is stabilized by the addition of ascorbic acid and which is capable of estimating concentrations of not less than $10 \mu\text{g./l.}$ Recovery of added adrenaline amounted to 80% and a factor was employed to correct for the loss. Lund did not find measurable

amounts of adrenaline in venous blood and concluded that adrenaline, if present at all, occurred in concentrations of less than $10 \mu\text{g./l.}$

Although the sensitivity of Lund's method could perhaps be increased by suitable modifications, the procedure seems to have other disadvantages. It is stated that adrenaline is quantitatively oxidized to adrenochrome by manganese dioxide without adrenochrome itself being further oxidized. The need for a rapid and complete removal of manganese dioxide is, however, emphasized and variations of such factors as temperature or the activity of the manganese dioxide preparation might, unless carefully controlled, entail the risk of a loss of adreno-

chrome. Secondly, as pointed out by Fischer, Derouaux, Lambot & Lecomte (1950), adrenochrome is liable to be reduced in the presence of ascorbic acid and may thus partly escape transformation to the fluorescent compound in alkaline solution. Whether these side reactions can seriously affect the practical value of the method has not been studied by us.

In the following, a fluorimetric method of adrenaline estimation will be presented which is not, like previous fluorimetric methods, based on the formation of adrenolutine (Lund, 1949a), but on a condensation with ethylenediamine. The reaction was discovered by Natelson, Lugovoy & Pincus (1949), but, as far as we are aware, it has not been further studied or applied to blood. The condensation takes place in alkaline solution in which adrenaline is readily autoxidizable. No reaction occurs if oxygen is excluded. If adrenaline is replaced by adrenochrome, a fluorescence intensity curve is obtained, both in presence and in absence of oxygen, which is identical with that produced by equimolar amounts of adrenaline in presence of oxygen. It may therefore be concluded that the condensation is preceded by an oxidation of adrenaline (I) to adrenochrome (II), and the reaction may be formulated as follows:



(I) Adrenaline. (II) Adrenochrome. (III) Ethylene diamine. (IV) Condensation product (hypothetical).

The constitution of the condensation product (IV) must at present be regarded as hypothetical.

The fluorescence of the final extract remains constant for at least 24 hr. The fact that the unstable, highly reactive adrenochrome is trapped in the nascent state and quantitatively converted into a completely stable condensation product is the most notable advantage of the method. In addition, the sensitivity is such that adrenaline concentrations of 1 $\mu\text{g./l.}$ can be measured with accuracy; the recovery is quantitative and the use of a correction factor is therefore unnecessary; and finally, strictly additive results are obtained with mixtures of adrenaline and noradrenaline, whereas the production and the decay of fluorescence observed in an alkaline

solution of adrenaline is disturbed by noradrenaline (Heller, Setlow & Mylon, 1950).

The procedure of Natelson *et al.* (1949) has been modified: the condensation is carried out by heating the solution at 50° for 20 min. with a mixture of ethylenediamine and an aqueous solution of ethylenediamine dihydrochloride. The free base is added to produce the required degree of alkalinity (pH of the mixture = 10.4) and may be replaced by ammonia. Since the presence of ethylenediamine in the ionized form is essential, it is obviously the reactive agent. For the extraction of the fluorescent condensation product *isobutanol* was used in preference to amyl alcohol; a quantitative extraction was achieved by saturating the aqueous phase with sodium chloride.

Attempts to replace ethylenediamine by *o*-phenylenediamine were abandoned owing to the formation of highly fluorescent oxidation products in the reagent blank.

The initial separation of adrenaline by chromatography introduced by Lund (1949b) has been adopted, with some modifications designed to reduce the autoxidation of adrenaline before and during the adsorption. Acid-washed alumina is used and its quantity is reduced. Another point

which was found to be particularly important is the removal of traces of heavy metal from the sodium acetate solution that is added to plasma prior to adsorption. This is done by passing the solution through a column of cation-exchange resin. Finally, the precise adjustment of the diluted plasma to pH 8.4 is controlled by a glass electrode.

The blood is drawn directly into a solution of fluoride and thiosulphate which combines anti-oxidant with anticoagulant properties. The apparent adrenaline content remains constant for at least 24 hr. at refrigerator temperature. In a few instances red blood cells were washed and cytolysed. Their apparent adrenaline content was about equal to that of plasma in confirmation of Lund's results,

but the alumina columns were liable to become clogged when haemolysates were used, and for this reason most estimations were carried out with separated plasma.

METHOD

Reagents

Sodium fluoride-sodium thiosulphate solution. NaF (2 g.) and $\text{Na}_2\text{S}_2\text{O}_3$ (3 g.), are dissolved in glass-distilled water to 100 ml. The solution is autoclaved and transferred to sterile ampoules, each containing about 6 ml.

Sodium carbonate. A 0.5N solution.

Sodium acetate buffered at pH 8.4. A 0.2M solution of sodium acetate is passed over a column of Zeo-Karb 215 (Permutit Co. Ltd.) and adjusted to pH 8.4 by the addition of 0.5N Na_2CO_3 (about 5 ml./l.) with the aid of a glass electrode. The Zeo-Karb column is prepared by repeated washings with 2N-HCl, water and 4% NaCl, the last being continued until the pH of the filtrate rises to about 6. Excess NaCl is flushed out with glass-distilled water.

Alumina, acid-washed. Al_2O_3 (100 g.; British Drug Houses Ltd., 'for chromatographic adsorption analysis') is stirred with 500 ml. boiling 2N-HCl for 20 min., filtered and washed on the funnel with 500 ml. of hot 2N-HCl. It is then repeatedly washed by decantation with distilled water and dried at 300° for 3 hr.

Acetic acid. A 0.2N solution.

Ethylenediamine dihydrochloride, 2M solution. The salt is prepared from freshly redistilled ethylenediamine by the slow addition of the calculated amount of 6N-HCl (A.R.). Crystals are filtered by suction, dried on a water bath and finally over H_2SO_4 .

Ethylenediamine, or ethylenediamine hydrate. Redistilled. *isoButanol.* A laboratory chemical grade is satisfactory.

Procedure

Fluoride-thiosulphate solution (5 ml.) is drawn up into a 20 ml. syringe and about 15 ml. blood collected from the cubital vein. The syringe is emptied into a 25 ml. measuring cylinder and the volume is noted (v_1 ml.). The plasma is separated by centrifugation (v_2 ml.) and an equal volume of sodium acetate buffer added. The mixture is brought to pH 8.4 by the addition of 3-5 drops of Na_2CO_3 solution, precise adjustment being controlled by a glass electrode.

Thistle funnels with a 50 ml. bulb and a 5 mm.-bore stem are used for the chromatographic separation. At a distance of about 18 cm. from the bulb, the stem has a constriction on which is placed a plug of glass wool. The funnel is mounted on a filter flask. The column is prepared by pouring in 0.7 g. of dry acid-washed alumina followed by 5 ml. acetate buffer. With the aid of a drawn-out glass rod the alumina is stirred up until all trapped air bubbles have escaped. After allowing it to settle, mild suction is applied which is so regulated that the rate of filtration does not exceed 20-30 drops/min. The plasma-acetate mixture is added next, followed by 5 ml. acetate buffer and 5 ml. glass-distilled water. The filtrates are rejected. The adsorbed adrenaline is eluted by passing 5 ml. 0.2N-acetic acid, followed by 5 ml. water, through the column.

After the addition of 0.5 ml. ethylenediamine dihydrochloride solution and 0.7 ml. ethylenediamine (or 1 ml. ethylenediamine hydrate) the eluate is heated at 50° for

20 min., cooled to room temperature and saturated with solid NaCl (about 4 g.). The solution is extracted with 6 ml. *isobutanol* in a mechanical shaker for 4 min. and lightly centrifuged to break up the emulsion. The *isobutanol* layer is removed completely and 5 ml. are measured into the fluorimeter tube. To avoid unnecessary transfer the eluate is collected in a glass-stoppered centrifuge tube in which the condensation and extraction procedures are carried out.

A standard containing 0.2 µg. adrenaline in 10 ml. and a reagent blank are carried through the condensation and extraction procedures together with the plasma eluates.

Measurement of fluorescence

A 125 W. high-pressure mercury vapour lamp with stabilized arc (Type MBL/D, British Thomson-Houston Co. Ltd.), operated in conjunction with choke and voltage stabilizer (Type MT140A, Advance Components Ltd.) serves as light source. The exciting light is passed through a blue filter (Chance OB10, 2 mm. thick) and focused by a thin-walled, flat-bottomed round flask of 250 ml. capacity, filled with water, on to a thin-walled test tube of non-fluorescent glass (11 mm. internal diameter). The test tube is held inside a light-tight box to which is attached, at right angles to the beam of the exciting light, the photocell compartment of a Unicam S.P. 500 spectrophotometer. The fluorescent light is filtered through a yellow filter (Chance OY4, 2 mm. thick) before entering the photocell. The photocell current is measured with the aid of the electrical controls provided by the Unicam spectrophotometer with slight modifications. It was necessary to change the bias on the first amplifier valve which controls the range of illumination at which the photocell works. This involved fixing a 100 000 Ω variable potentiometer between two taps on battery no. 3 of the instrument, and this control was fitted with a built-in switch to return the instrument to its normal operating state when used as a spectrophotometer.

Readings are taken 45 min. after switching on the mercury lamp in order to eliminate drift during the warming-up period. The reagent blank is used to establish the zero position of the 'Transmission' control: by means of the new control for rough adjustment and the 'Dark Current' control for fine adjustment the potential of the photocell at this illumination level is backed-off until balance is obtained with the 'Transmission' dial set at zero. The standard is now introduced into the fluorimeter box, and with the 'Transmission' dial at 100, balance is obtained by operating the 'Sensitivity' control. Next the unknown sample is inserted and balance is established by turning the 'Transmission' control. The position of the dial shows the intensity of the unknown fluorescence as a percentage of the standard fluorescence. When a reading is taken, the shutter which normally protects the photocell is opened and replaced as soon as the necessary adjustments have been made so as to avoid undue fatigue. In each series of readings the position of the zero and the 100 setting is checked at intervals by re-introducing the reagent blank and the standard, respectively. When a series has been completed, the readings are repeated in the same order. Duplicate readings obtained in this way may differ somewhat owing to instabilities in the amplifier circuit or to fluctuations of the mercury lamp output. The mean of the duplicate readings is used to calculate the result.

Calculation

If the fluorimetric measurement indicates the presence of a $\mu\text{g.}$ adrenaline, the amount, per litre of plasma, is

$$\frac{a \times v_1 \times 1000}{v_2(v_1 - 5)} \mu\text{g.}$$

RESULTS AND DISCUSSION

Choice of standard

When a standard of 0.2 $\mu\text{g.}$ adrenaline is used to adjust the sensitivity setting the calibration curve obtained is linear (Fig. 1), indicating proportionality between fluorescence intensity and photocell current.

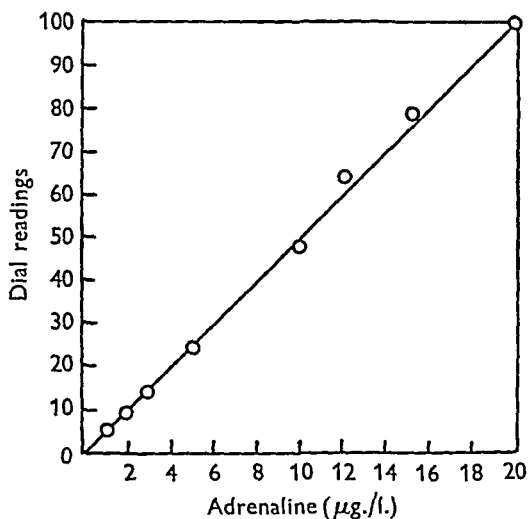


Fig. 1. Calibration curve of fluorimetric estimation of adrenaline.

This setting was optimal for the equipment available. Attempts to increase the sensitivity to the point where a dial reading of 100 was obtained with a standard of 0.1 $\mu\text{g.}$ resulted in a calibration curve which was no longer linear; in addition, there was a disproportionate loss of discrimination. The sensitivity setting obtained with a standard of 0.2 $\mu\text{g.}$ proved satisfactory for our purpose, since with most blood samples the scale readings were between 15 and 20 on a scale of 100 divisions. If it should be required, the sensitivity could no doubt be increased, either by changing the type of photocell, or by intensifying its illumination.

Error of fluorimeter readings

On the scale of 100 divisions, 1 division corresponds to 0.002 $\mu\text{g.}$ adrenaline, given the usual sensitivity setting. Readings were taken to 0.1 of a division, the last figure being obtained by approximate estimation. Owing to slight instabilities in the set-up, duplicate readings differed to some extent. The standard deviation of single readings was calculated

from the sum of the squares of the differences (Δ) between n pairs of duplicate readings according to the formula $s = \sqrt{\frac{\sum(\Delta^2)}{2n}}$. The results show that the error does not appreciably vary in different ranges of dial readings, the standard deviation being close to 0.5 division throughout (Table 1). This indicates that single readings are accurate to approximately ± 1 division and the mean of duplicate readings to approximately $\pm 1/\sqrt{2} = 0.7$ division, or 0.0014 $\mu\text{g.}$ adrenaline.

Table 1. Error of fluorimeter readings

No. of samples	Range of dial readings (divisions)	Standard deviation of single reading (divisions)
31	5-10	0.406
67	10-15	0.437
45	15-20	0.388
27	20-40	0.601
15	40-70	0.482

Recovery

Both adrenaline and noradrenaline were quantitatively recovered when pure solutions were carried through the chromatographic adsorption process. The recovery from plasma was studied by adding varying amounts of adrenaline to 10 ml. samples of sterile horse serum. The results of ten such experiments showed that recovery was quantitative with a standard deviation of about 5% over the entire range investigated (Table 2). To be significant at a probability level of 0.01, the difference between two results must exceed 15% or, at a probability level of 0.05, 11%.

Table 2. Recovery of adrenaline added to horse serum (Ten experiments for each quantity of adrenaline added.)

Adrenaline added ($\mu\text{g./10 ml.}$)	Mean recovery (%)	Standard deviation of recovery
0.02	100.55	6.34
0.03	100.01	4.70
0.05	97.89	4.90
0.10	97.34	4.47

Equally good recoveries, obtained by different methods, have been reported by many other authors. Nevertheless, the estimates of the normal level of blood adrenaline differ widely according to the method used. Obviously, a satisfactory recovery of added adrenaline excludes an underestimate, but not an overestimate. This can only be done by obtaining further evidence.

Specificity

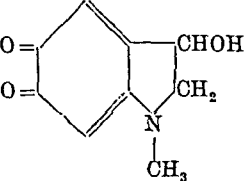
Fluorescence of variable intensity is produced by all catechol derivatives tested, with the exception of dihydroxyphenylalanine (Table 3). This presumably forms a derivative which is not extracted

by *isobutanol* from the alkaline solution, or only to a slight extent. None of the non-catechol substances tested produced any significant fluorescence after their solutions had been carried through the chromatographic adsorption process.

The reaction is thus less specific than that resulting in the formation of adrenolutine or similar com-

diamine was omitted and plasma eluates were directly extracted with *isobutanol*, the fluorescence of the extract was smaller than that of the usual reagent blank. This indicates that none of the fluorescence measured in the usual test is due to preformed plasma components, but is produced entirely during the condensation reaction.

Table 3. *Specificity of method*

<i>Catechol derivatives</i>	Formula	Relative fluorescence
L-Adrenaline	(3) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NHCH}_3$ (1) (4) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NHCH}_3$	100
Adrenochrome		100
L-Noradrenaline	(3) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NH}_2$ (1) (4) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NH}_2$	20
Epinephrine	(3) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NHCH}_3$ (4) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NHCH}_3$	48
Isoprenaline	(3) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NHCH}(\text{CH}_3)_2$ (4) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{NHCH}(\text{CH}_3)_2$	56
Catechol	(1) $\text{HO}-\text{C}_6\text{H}_4$ (2) $\text{HO}-\text{C}_6\text{H}_4$	96
L-Dihydroxyphenylalanine	(3) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CH}_2\cdot\text{CHNH}_2\cdot\text{CO}_2\text{H}$ (4) $\text{HO}-\text{C}_6\text{H}_3\cdot\text{CH}_2\cdot\text{CHNH}_2\cdot\text{CO}_2\text{H}$	0.2
<i>Other compounds</i>		
Ephedrine	$\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}(\text{NH}_2)\text{CH}_3$	2×10^{-4}
Amphetamine	$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\text{CH}_3$	1×10^{-6}
Tyramine	(p) $\text{HO}\cdot\text{C}_6\text{H}_4\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$	3×10^{-3}
Resorcinol	(1) $\text{HO}-\text{C}_6\text{H}_4$ (3) $\text{HO}-\text{C}_6\text{H}_4$	~ 0
Quinol	(1) $\text{HO}-\text{C}_6\text{H}_4$ (4) $\text{HO}-\text{C}_6\text{H}_4$	~ 0
Nicotinamide	$\text{C}_5\text{H}_4\text{N}\cdot\text{CONH}_2$	~ 0

pounds, for which indole ring closure is required. But this is of no practical consequence since physiological catechol derivatives which are not capable of indole ring closure, such as dihydroxyphenylalanine and, possibly, dihydroxyphenylserine, are largely eliminated by the extraction procedure.

When the condensation reaction with ethylene-

The fluorescence intensity produced by noradrenaline is one-fifth that produced by an equimolar amount of adrenaline. The fluorescence produced by a mixture of both substances is equal to the sum of the fluorescence produced by the separate components. There is thus no mutual interference like that observed under different conditions by Heller *et al.* (1950).

Rate of autoxidation

When plasma was diluted with an equal amount of sodium acetate buffer, adjusted to pH 8.4, and shaken in air, added adrenaline was found to disappear at about the same rate as the preformed material (Table 4). To facilitate autoxidation, sodium thiosulphate was omitted from the anticoagulant solution and the sodium acetate solution used in the experiment was not previously purified by cation exchange.

The experiment shows that in its autoxidizability the preformed substance behaves like adrenaline.

Table 4. Autoxidation of preformed and added adrenaline in human plasma

(Blood (150 ml.) collected in 50 ml. 2% NaF (no Na₂S₂O₃.)

Adrenaline (μg./l.)			
Found			Loss (%)
Added	Initial	After 30 min. shaking in air (37°, pH 8.4)	
0	3.1	1.6	48.5
20	22.9	9.8	57

Action of amine oxidase

It could further be shown that an extract of rabbit liver removed preformed material and added adrenaline at an equal rate from plasma (Table 5). The extract was inactivated by boiling and inhibited by ephedrine, a specific inhibitor of amine oxidase (Blaschko, Richter & Schlossmann, 1937). The inhibition by ephedrine was the same whether the enzyme acted on the preformed substance or on added adrenaline. It may therefore be concluded that the action of the liver extract was due to its content of

amine oxidase, and that the substance preformed in plasma is an amine derived from catechol with affinities for amine oxidase similar to those of adrenaline.

It remained to be shown that the entire fluorescence measured in the test was due to this substance and was not the resultant of several components. It was found that amine oxidase

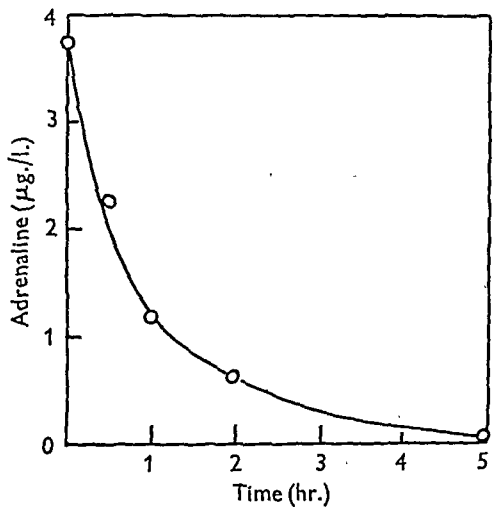


Fig. 2. Action of amine oxidase on preformed reacting material in human plasma. Enzyme prepared as in experiment of Table 5. Plasma (15 ml.) incubated with 3 ml. liver extract at 37°. Results were corrected by subtracting the fluorescence obtained in controls containing 15 ml. water + 3 ml. liver extract.

quantitatively removes the preformed material from plasma (Fig. 2). The plot is that of a first-order reaction, i.e. a straight line is obtained on a semi-logarithmic scale. The bulk of the reacting material is therefore either a single substance or a mixture of substances with similar affinities for the enzyme.

Table 5. Action of amine oxidase on preformed and added adrenaline in plasma

(Enzyme: rabbit liver homogenized with 1 part water and centrifuged. Residue washed with 1 part water. Combined supernatants dialysed against running distilled water at 0° for 16 hr. Blood (150 ml.) collected in 50 ml. NaF-Na₂S₂O₃ solution. Samples contained 15 ml. plasma, with or without 3 ml. liver extract. Incubation at 37° for 2 hr.)

		Adrenaline found (μg./l.)	
		Preformed	After adding 10 μg./l.
Sample no.	Addition to plasma		
S1	0	3.7	11.2
S2	0.01 M-Ephedrine	4.0	11.7
S3	Liver extract, boiled	4.3	13.0
S4	Liver extract + 0.01 M-ephedrine	3.4	10.1
S5	Liver extract	1.4	4.1
Disappearance (S3 - S5)		2.9	8.9
Percentage disappearance $\left(\frac{(S3 - S5)}{S3} \times 100\right)$		67.5	68.5
Percentage inhibition by ephedrine $\left(\frac{(S4 - S5)}{(S3 - S5)} \times 100\right)$		69	67.5

*The identity of the adrenaline-like substances
in blood*

It is now generally accepted that a mixture of adrenaline and noradrenaline, in varying proportions, occurs not only in the adrenal medulla, but in all tissues containing functional post-ganglionic sympathetic fibres, and that it is discharged into the blood stream when these fibres are stimulated (cf. references in a review by von Euler, 1950). The sympathomimetic amine occurring in human and cattle blood is, according to von Euler & Schmiterl w (1947), largely L-noradrenaline with an admixture of about 4 % adrenaline; rabbit blood, on the other hand, contains mainly adrenaline, according to West (1947). A third sympathomimetic amine, hydroxytyramine, may be the physiological precursor of noradrenaline (cf. Blaschko, 1950). Traces have been found in the adrenals (Goodall, 1950) and in urine (Holtz & Credner, 1942; von Euler & Hellner, 1951), but its concentration in blood is probably negligible.

Chemical, biological and chromatographic methods are available to distinguish between these amines, but these methods are generally not sensitive enough for application to the low concentrations occurring in blood. Thus the measurable limits which can be attained in the set of five pharmacological tests devised by Gaddum, Peart & Vogt (1949) are given as 10 $\mu\text{g./l.}$ for adrenaline and 100 $\mu\text{g./l.}$ for noradrenaline.

Nevertheless, at our request, Dr T. B. B. Crawford of the Department of Pharmacology, University of Edinburgh, applied the rat-uterus method to a plasma eluate which we had sent. Its apparent adrenaline content had previously been estimated by our method and amounted to 3.0 $\mu\text{g./l.}$ In the rat-uterus test adrenaline causes a depression of the contraction elicited by carbachol. The plasma eluate had a small effect in the wrong direction, since the response to carbachol was slightly increased. The addition of adrenaline to the eluate in a concentration of 2.5 $\mu\text{g./l.}$ was more than enough to antagonize the slight stimulant action. A reagent blank did not affect the definite response of the rat uterus to a solution containing 2.5 $\mu\text{g.}$ of adrenaline/l.

In our opinion, this result does not yet definitely exclude the identity of the plasma substance with adrenaline. As pointed out by Gaddum *et al.* (1949), blood contains interfering substances whose effect on the rat uterus is antagonistic to that of adrenaline. The eluate tested was not free of this interference and it is conceivable that the amount of preformed adrenaline was just adequate to counterbalance most of the antagonistic effect. It is, however, far more probable, especially in view of the results of von Euler & Schmiterl w (1947), that the bulk of the plasma substance is identical with noradrenaline rather than adrenaline. Work is in progress

to decide this problem by paper-chromatographic methods.

For many questions of clinical interest relative figures for the combined level of sympathomimetic amines in blood would be of value, provided there are no drastic changes in the proportion of the components. Such figures can be obtained in the clinical laboratory by the method described. Pending a final identification of the reacting material the results have been expressed in terms of adrenaline. To convert them to noradrenaline concentrations the figures have to be multiplied by 5.

*The concentration of adrenaline-like substances
in human blood*

Fifty-four samples of blood were collected from the staff and patients of Runwell Hospital under ordinary conditions. The mean amine concentration corresponded to $3.39 \pm 0.043 \mu\text{g.}$ adrenaline/l. plasma. Variations from the mean were comparatively slight, the range being from 2.9 to 4.3 $\mu\text{g./l.}$

The values for the normal adrenaline content of blood reported by previous authors are notoriously divergent (for reviews of the literature see Lehmann & Michaelis, 1942; J rgensen, 1945; Blaschko, 1950). Most of them are much higher than those found by our method, a clear indication that the methods used were lacking in specificity. There are, however, some results, such as those of Bloor & Bullen (1941) and especially those recently obtained by Anrep, Barsoum & Gabrawy (1950), which broadly agree with ours, or such as those of Lund (1949b) which, at least, do not contradict them. Expressed as L-noradrenaline our results are in remarkably good agreement with the value of 10–20 $\mu\text{g./l.}$ found by von Euler & Schmiterl w (1947).

Some authors (Stewart & Rogoff, 1917; Trendelenburg, 1923; Guyton & Gillespie, 1951) tried to calculate the circulating concentration of adrenaline from a consideration of the rates of secretion and elimination or the amounts required to maintain blood pressure after adrenalectomy. They all arrived at figures in the neighbourhood of 1 $\mu\text{g./l.}$ It seems that, in order of magnitude, these estimates which neglected extra-adrenal sources of sympathomimetic amines are confirmed by our observations.

The extremely high values obtained by Lehmann & Michaelis (1942, 1949) and by Annersten, Gr nwall & K iw (1950) in non-deproteinized plasma are attributed by these authors to a protein-bound, biologically inactive form of adrenaline. For this there is at present no experimental support. As pointed out by Lund (1949b), it is unlikely that the adrenaline-protein complex would remain undissociated when plasma is passed through an alumina column. There is certainly no retention of adrenaline when it is added to plasma.

*Changes of plasma adrenaline concentration
under various conditions*

Electrical stimulation of the brain by external electrodes may increase the apparent adrenaline level in plasma by as much as 100 %, whether convulsions occur or not. In general anaesthesia, on the other hand, the adrenaline content may fall to about 40 % of the initial value.

Contrary to a widely held opinion a highly significant fall of the adrenaline level in blood is invariably observed during insulin hypoglycaemia. This phenomenon is fully developed within 30 min. after the intramuscular injection of insulin, long before consciousness becomes clouded. The effect is not due to an exhaustion of the adrenergic system, since an intravenous injection of glutamic acid, or the conduction of electric current through the brain, during hypoglycaemic coma, causes a pronounced rise of blood adrenaline.

These effects will be fully reported in a later publication.

SUMMARY

1. A fluorimetric method for the estimation of adrenaline-like substances in blood is described. It consists of the following steps: (a) filtration of plasma-buffer mixture (pH 8.4) through a column of acid-washed alumina and elution of the adsorbed amines by dilute acetic acid; (b) heating of the eluate at 50° with a mixture of ethylenediamine and ethylenediamine dihydrochloride; (c) extraction of a stable fluorescent condensation product with

isobutanol; (d) measurement of fluorescence. The method has the advantage, compared with previous methods, that adrenochrome, a labile oxidation product of adrenaline formed as an intermediary, is trapped in the nascent state and quantitatively converted into a stable condensation product.

2. A series of recovery experiments showed that added adrenaline was quantitatively recovered with a standard deviation of about 5 %. Concentrations $\geq 1 \mu\text{g./l.}$ may be determined by the method.

3. A study of the specificity of the method and of the action of amine oxidase led to the conclusion that the reactive material in blood consists entirely of amines derived from catechol. Whether this is adrenaline, noradrenaline or a mixture of both, remains to be further investigated.

4. Expressed in terms of adrenaline the mean concentration observed in human venous blood under normal conditions is about $3 \mu\text{g./l.}$ Since the fluorescence formed from noradrenaline is one-fifth of that produced by adrenaline, figures of adrenaline concentrations have to be multiplied by 5 to convert them to noradrenaline concentrations.

5. Some further results obtained with the method are briefly reported.

We are greatly indebted to Prof. J. H. Gaddum, F.R.S., and to Dr T. B. B. Crawford for their co-operation in carrying out pharmacological tests on a plasma eluate, and to Mr N. W. Ellis for help and advice concerning the adaptation of the Unicam spectrophotometer to fluorimetry. We also wish to acknowledge a gift of L-noradrenaline by Messrs Bayer Products Ltd. and of epinine and isoprenaline by the Wellcome Foundation Ltd.

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The Corneal Mucopolysaccharide

By A. M. WOODIN

*Ophthalmological Research Unit (Medical Research Council)
Institute of Ophthalmology, London, W.C. 1*

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The acid mucopolysaccharide of cornea is present in the ground substance between the collagen fibres of the stroma. Although the presence of such a substance has been known since the work of Morner (1894) its chemical nature has been the subject of much discussion and the relation between the mucopolysaccharide and the collagen fibres has not been considered. Such information is of value for many reasons, amongst which can be mentioned the control of the permeability of the stroma, and the possible dependence of the transparency of the tissue on the relation between the mucopolysaccharide and the collagen fibres.

Meyer & Chaffee (1940), who summarized the earlier work on the corneal mucopolysaccharide, obtained a protein-free preparation from an extract of the water-insoluble residues of cornea. This preparation gave positive reactions for a uronic acid, for acetyl groups, and for ester-S; glucosamine hydrochloride was isolated from a hydrolysate. These substances were present in equimolecular amounts. Moreover, they found that the corneal mucopolysaccharide was a substrate for enzyme preparations having hyaluronidase activity, and they therefore suggested that it was a natural sulphuric acid ester of hyaluronic acid. The optical rotation was in keeping with this hypothesis, and they called the corneal mucopolysaccharide hyaluronosulphate.

Wislocki, Bunting & Dempsey (1947) found that the intensity of metachromasia in sections of cornea stained with toluidine blue was not reduced by treatment of the sections with hyaluronidase, but drew no conclusions from the observation. In a further paper (Dempsey, Bunting, Singer & Wislocki, 1947), they described the basophilia-pH curve of various tissues containing mucopolysaccharides. That of the cornea resembled those for tissues containing hyaluronic acid, in having the basophilia suppressed at pH 4. As an interpretation they suggested that the ester-S groups were not ionized in the intact tissue, or that they were absent from the mucopolysaccharide.

Werner & Odin (1949) analysed by paper chromatography a mucin from the aqueous extract, and an acid mucopolysaccharide obtained from the water-insoluble residues of cornea. Both preparations contained glucosamine, glucuronic acid,

galactose, mannose and ester-S. Glucose was present in the digest from the aqueous residues but not in the mucin. They suggested that the cornea contains both a neutral and an acid mucopolysaccharide. It is difficult to evaluate their results as it is apparent that neither of their preparations was homogeneous and, although they suggested a progressive liberation of monosaccharide residues on acid hydrolysis, they did not report the position on their chromatograms of products of partial hydrolysis.

Evidence for the inactivity of hyaluronidase in the cornea was offered by Woodin (1950a), who found that enzyme preparations from several sources were without action as spreading factors, even under artificial conditions chosen to facilitate diffusion.

In this investigation, the stages in the extraction and purification of the mucopolysaccharide have been followed by a chemical and electrophoretic analysis in an attempt to determine the way in which the mucopolysaccharide is combined in the intact tissue. The action of hyaluronidase on the mucoids and mucopolysaccharide has been investigated, and a preliminary analysis of the mucopolysaccharide by paper chromatography has been attempted. Evidence so obtained shows that the corneal mucopolysaccharide cannot be described as a sulphuric acid ester of hyaluronic acid. It has been found further that the mucopolysaccharide is capable of forming complexes with proteins present in the tissues and it appears that, *in situ*, the mucopolysaccharide is in combination with proteins of the collagen group.

METHODS

Analytical methods

The analysis figures given in this paper refer to the dry, ash-free, material. The ash content of solids was determined after incineration with H_2SO_4 . When solutions were analysed they were dialysed against 0.001 N-HCl and subsequently against distilled water. The dry weight was then determined by drying at 100° and it was assumed to be ash-free. In some cases this was confirmed by ashing.

Total nitrogen. Kjeldahl digestion for 8 hr. with a selenium catalyst was followed by distillation in the Markham still. The distillate was collected in 0.01 N-Ba(OH)₂ and titrated against 0.01 N-HCl with a mixed methylene blue-methyl red indicator.

Ester sulphate was determined by the gravimetric procedure of Lugg (1938) after hydrolysis in 5.8N-HCl at 100° for 20 hr.; about 10 mg. of BaSO₄ were weighed and duplicates differed by 10%.

Hexosamine. This was determined by the modified Elson-Morgan reaction described by Pirie (1949). In the condensation reaction with acetyl-acetone the necks of the tubes were cooled with running water. Analytical figures given here refer to all the material which reacts with the Elson-Morgan reagents. In the early stages of this work, reported by Woodin (1950*b*), glucosamine standards were incorporated after the hydrolysis, but Ogston & Stanier (1950) have shown that glucosamine is destroyed when it is heated in acid solution and this result has been confirmed here. All results reported in this paper were obtained from standards which had been put through the hydrolysis procedure given to the corneal material.

pH 4-5 with Ba(OH)₂, and evaporated to dryness. About 300 µg. of the neutralized hydrolysate were added to the chromatogram. In some cases the neutralized hydrolysate was treated with ion-exchange resins before being added to the papers. Zeo-Karb 215 and De-acidite E were ground to a 40-mesh powder and 20 ml. wet vol. of each put into columns. The resins were washed and then regenerated with HCl and NH₄OH, respectively. The neutralized hydrolysate was diluted and 3 ml., containing 6 mg. of solid, pressed into the column. The non-absorbed sugars were washed out with 20 ml. water. A standard solution of galactose, glucosamine, and glucurone was put through the columns as a control.

When the amino-acid composition of the mucoids was required, they were hydrolysed in 6N-HCl for 12 hr. and the acid removed *in vacuo*. Two-dimensional chromatograms were run in phenol-NH₃-KCN and in collidine. They were developed with ninhydrin.

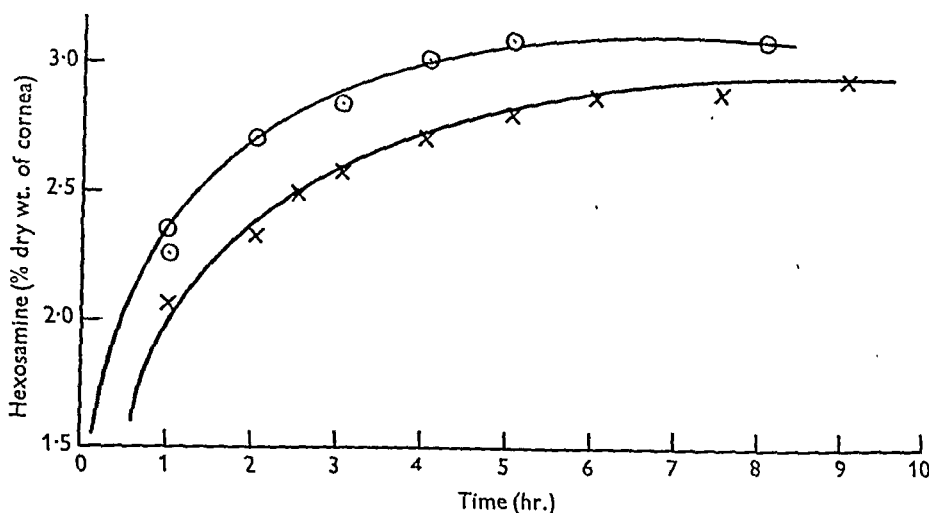


Fig. 1. Liberation of hexosamine from cornea on acid hydrolysis. ○, 5.8N-HCl; ×, 3.48N-HCl.

Fig. 1 shows the rate at which hexosamine is liberated from the cornea on acid hydrolysis. As a routine method the corneal material was hydrolysed in 5.8N-HCl for 6 hr. at 100°.

Electrophoresis

This was done in the Tiselius apparatus at +2° with the optical system of Philpot (1938). The conductivity of the buffer solutions was measured at 0°. The pH was measured at 0° by a glass electrode calibrated against 0.05M-phthalate buffer, pH 4.0. To reduce boundary anomalies and viscosity effects, solutions containing a high concentration of salt and a low concentration of colloid were used. The corneal material was dialysed against a buffer containing NaCl (1NaCl 0.18, 1 buffer 0.02) and then the solution diluted to give a refractive increment of 0.75×10^{-3} .

Chromatography on filter paper

The techniques and purification of solvents were those described by Partridge (1948*a*). The chromatograms were developed with aniline hydrogen phthalate (Partridge, 1949), the Elson-Morgan reagents (Partridge, 1948*a*) and with the AgNO₃-KOH reagent (Trevelyan, Proctor & Harris, 1950). The mucopolysaccharide was hydrolysed in N-H₂SO₄ for 6 hr. or in 5N-H₂SO₄ for 5 hr. The hydrolysates were brought to

Ionophoresis on filter paper

The apparatus described by Durrum (1950) was constructed and a 'power pack' used as the source of the current. Samples of the hydrolysate and of the standard sugars were put in line along the centre of a sheet of filter paper and the sheet draped over a glass rod with the line of spots as the apex. The apex was 25 cm. above the level of the electrolyte. A potential of 400-600 V. was then applied across the paper. After drying, the papers were developed with the aniline hydrogen phthalate, or the AgNO₃-KOH, reagents. No interference with the developers was found with 0.01N-NH₃, 0.01M-KH₂PO₄, 0.01N-Na₂HPO₄ or 5N-acetic acid, as electrolyte. In some cases the hydrolysate was first run as a partition chromatogram and then, after removing the solvent and adding the electrolyte, a potential was applied at right angles to the direction in which the chromatogram had been irrigated.

Hyaluronidase preparations

The enzyme preparations have been described previously by Woodin (1950*a*). The activity of the corneal mucopolysaccharide as substrate for hyaluronidase was assessed by measuring the reducing power of the solutions (Hagedorn & Jensen, 1923) after incubation with the enzyme. The solu-

tions of the corneal material were dialysed against a phosphate buffer containing NaCl (0.11M-NaCl, 0.02M-phosphate) before incubation. The pH of the buffer was 4.6, 5.5, 7.0, respectively, when testis, *Cl. welchii*, and streptococcal hyaluronidase, was used. The concentration of the corneal mucoids and mucopolysaccharide was deduced from the hexosamine content of the dialysed solutions.

Test of enzyme digestion

The dialysed substrate solution (2 ml.) was put in tubes and 0.1 ml. of the enzyme added. The mixed solution (0.5 ml.) was immediately withdrawn and added to the Hagedorn-Jensen reagent. The rest of the solution was incubated at 37° (see Table 5) and then 0.5 ml. withdrawn and added to the Hagedorn-Jensen reagent.

A crude preparation of hyaluronic acid from ox vitreous filtrate was digested in parallel. The filtered vitreous was precipitated with 3 vol. of acetone containing 3% acetic acid, and the precipitate collected, suspended in water, and dialysed. The insoluble material was rejected and the supernatant precipitated again with acid acetone. The precipitate was then dried. After dialysis against the appropriate buffer, the concentration of the hyaluronic acid was determined from the hexosamine liberated after hydrolysis in 5.8N-HCl for 6 hr. at 100°.

Viscosity measurements

These were made at 25° in an Ostwald viscosimeter having a flow time of 20 sec. for 3 ml. water. The corneal mucoids were dialysed against a phosphate-NaCl buffer at pH 7 (*I*NaCl 0.18, *I*phosphate 0.02). The concentration of the solutions was determined from their nitrogen content. For each substance investigated, a curve was constructed relating the concentration to the log. of the relative viscosity. This relationship was linear and in the section describing the results the viscosity of the mucoids will be given as log η_{rel} for a 0.5% solution.

Preparation of material

The corneas were cut from ox eyes 2-3 hr. after the death of the animal, rinsed in distilled water, and frozen into blocks. They were then cut to 20 μ . sections on the freezing microtome. The sections could then be dried by sublimation, or by acetone, or could be extracted directly.

Extraction of the cornea

In general a known weight of the sections (of which the dry weight and ester-S content was known) was suspended in the solvent using about 25 ml. for each gram, dry weight, of the sections. The suspension was brought to pH 8, left for 3-4 days at 0°, and then centrifuged and the residues extracted again twice. Finally the residues were dialysed till free from Cl- and their dry weight and ester-S content determined.

Sulphatase activity

If the extraction experiments were to be of value, it was important to know whether the corneal sections had a sulphatase activity. 1 g. sections, dried by sublimation, was suspended in water, the pH of the suspension being 6.7, and then incubated at 37° in the presence of toluene for 24 hr. A further sample was heated to 100°, 25 ml. of boiling water added and the suspension kept at 100° for 15 min.,

then cooled and incubated at 37° for 24 hr. At the end of the incubation period the tubes were filtered, the filtrates acidified and the mucin was centrifuged off. 0.5M-BaCl₂ (1 ml.) was then added to 15 ml. of the supernatant. A visible precipitate of BaSO₄ was produced in each case, but neither weighed more than 200 μ g.

RESULTS

Composition of the cornea. The sections of cornea, after washing in acetone, contained 0.25-0.26% ester-S and 3.00-3.25% hexosamine. The ester-S corresponds to a mucopolysaccharide content of 4.2%. On the basis of their final yield, Meyer & Chaffee (1940) gave the mucopolysaccharide content of the cornea as 1.8%. Only 54% of the hexosamine of the cornea is accounted for as a component of the mucopolysaccharide. Under the conditions of the test, the corneal sections had no sulphatase activity. The inorganic sulphate in the cornea represents less than 5% of the ester sulphate.

Extraction of the mucoids from cornea. Meyer & Smyth (1937) used 10% calcium chloride to extract acid mucopolysaccharides from connective tissues. Partridge (1948*b*) obtained good yields of chondroitin sulphate by extracting cartilage with 10% calcium chloride. Extraction with sodium chloride at pH 8 is the basis of the Bergman & Stein (1939) method of preparing collagen and was used by Pirie (1947) to free corneal collagen from the ground substance. These methods have been compared

Table 1. Extraction of cornea at pH 8

(Where sections were extracted successively by different solvents the amount of mucoid dissolved, given in column 3, is the percentage of that initially present in the intact tissue. The amount in the residues is that remaining after extraction with all solvents.)

Nature of sections	Solvent	Ester-S dissolved (%)	Ester-S in residues (%)
Dry	10% CaCl ₂	45-50	0.2
Wet	10% CaCl ₂	60-65	0.1
Wet	(a) 10% NaCl	40	—
	(b) 10% CaCl ₂	30	0.09
Wet	(a) Water	20	—
	(b) 10% NaCl	30	—
	(c) 10% CaCl ₂	35	0.05
Swollen	10% CaCl ₂	85	0.06

here. Initially the tissue was dehydrated with acetone to denature water-soluble proteins, but it has been found that drying the tissue reduces the efficiency of the extraction. A better yield was obtained from sections which had not been dried. The most efficient extraction was from sections which had swollen in water to give a wet weight/dry weight ratio of 32.

The results of the extraction experiments are given in Table 1. The same efficiency of extraction of

dry sections was found whether drying was by acetone at room temperature or at -10° , or by sublimation. Heating the sections at 45° for 30 min. (Partridge, 1948b) did not increase the yield of mucoid.

More mucoid could be dissolved from the residues after extraction at pH 8 by heating them at 37° and pH 11 for 30 min. Although a mucoid rich in ester-S could be recovered by fractionation of this extract, the residues from the extraction at pH 8 and at pH 11 contained the same amount of ester-S. It appears that protein and mucopolysaccharide are dissolved to the same extent at pH 11.

ethanol at -10° . These substances will be called the 'insoluble mucoids', and the 'soluble mucoids', respectively. The insoluble mucoids were washed with water and the suspension frozen and dried by sublimation. The soluble mucoids were dissolved in water and dried by sublimation.

We shall be concerned with the extracts made from acetone-dried sections, from water-swollen sections, and from the residues remaining after extraction of the sections with water. Only the extracts made in calcium chloride solution have been studied to any extent. The soluble mucoids and the insoluble mucoids will be described separately. The soluble

Table 2. *Composition of corneal extracts*

	N (%)	Ester-S (%)	Hexosamine (%)
(1) Extract made in distilled water	13.1	0.53	7.3
(2) Direct precipitation with 3 vol. ethanol and 0.5% sodium acetate of extract made in 10% CaCl_2 :			
From dry sections pH 8	13.4	0.70	8.8
From fresh sections pH 8	12.3	0.70	9.0
From fresh sections pH 8 with heat treatment	14.6	—	7.5
From fresh sections pH 11 (after pH 8 extraction)	14.8	0.80	5.0
(3) Water-soluble mucoids prepared by 1 vol. ethanol precipitation of dialysed CaCl_2 extract, pH 8:			
From dry sections	11.5	1.1	11.4
From swollen sections	11.4	1.3	11.7
From water-extracted sections	11.2	2.1	12.7
(4) Water-soluble mucoids from dialysed CaCl_2 extract, not fractionated with ethanol:			
From dry sections	12.2	0.73	8.2

Table 3. *Electrophoresis of soluble mucoids*

(The refractive increment of the solution was about 0.75×10^{-3} in a buffer of composition 1 NaCl 0.18, 1 buffer 0.02. A current of 0.01–0.02 amp. was passed for 6–8 hr.)

Origin	pH	No. of components	Contribution of fastest to total refraction (%)	Hexosamine/nitrogen ratio of fastest component
Dry sections	9.05	2	60	1.3
	6.80	2	62	1.3
Swollen sections	6.8	4	50	1.4
	4.7	3	50	—
Water-extracted sections	6.8	2	62	2.5

Fractionation of the extracts. In the earliest stages of this work the extracts were precipitated directly with 3 vol. ethanol and sufficient sodium acetate to give a final concentration of 0.5%. The solid obtained had a high ash content and was only partly soluble in water and dilute salt solutions; moreover, it gave unsatisfactory results when fractionated by the methods described below. The composition of the products of direct precipitation is given in Table 2.

Subsequently, the extracts made in salt solutions were dialysed against running tap water and then against distilled water. A precipitate appeared during the dialysis and material remained in solution which could be recovered by the addition of 1 vol. of

mucoids account for 85% of the total amount of ester sulphur extracted from the swollen sections, and for 80% of the total extracted from the dry sections. Fig. 2 summarizes the fractionation procedures described below.

The soluble mucoids

The composition of the soluble mucoids is given in Table 2. Solutions of the soluble mucoids were examined in the Tiselius apparatus since it was hoped to prepare a protein-free preparation of the mucopolysaccharide by the electrophoretic method. However, as Table 3 indicates, the fast components of the mucoids always contain considerable amounts

of protein. The mobility of the slowest component was very low and these results are not evidence for its homogeneity.

represent part of the water-soluble proteins of the cornea. They are absent from the extract from dry sections, perhaps because of denaturation by the

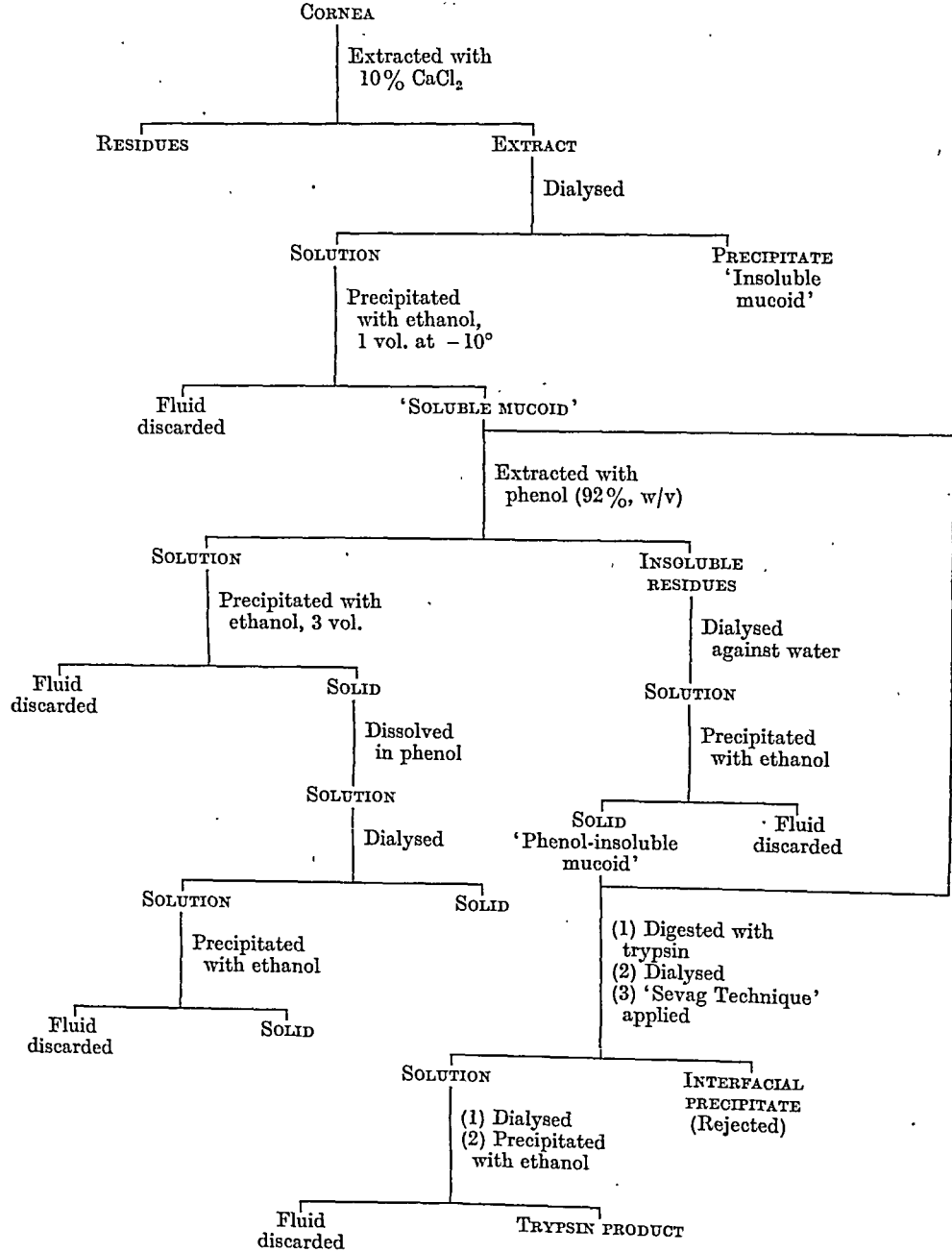


Fig. 2. Diagram of fractionation procedure.

At pH 4.7 the fast component of the soluble mucoids from the swollen sections contained 11.0 % nitrogen and the other components contained 12.0 % nitrogen. The two components of intermediate mobility present in this extract probably

acetone, and from the soluble mucoids from the aqueous residues because of the preliminary water extraction. Preliminary experiments on the water-soluble fraction of the corneal proteins support this suggestion. The aqueous extract had four com-

ponents at pH 6.8, the fastest being responsible for 20 % of the total refraction and having a mobility of the same order as that of the soluble mucoids from dry and swollen sections. When the aqueous extract was precipitated with 3 vol. of ethanol and 0.5 % sodium acetate at -10° , the precipitate did not dissolve when suspended in water and dialysed.

The mobilities of the fast components of the mucoids is given in Table 4.

Table 4. *Electrophoretic mobilities of fast components of the mucoids*

(The electrolyte had the composition 1 NaCl 0.18, 1 buffer 0.02. The mobilities are means from both limbs.)

Origin	Buffer	pH	Mobility $\mu \times 10^5$ (cm. ² sec. ⁻¹ V. ⁻¹)
Soluble mucoids from acetone-dried sections	Glycine	9.05	-7.6
	Phosphate	6.80	-7.4
Soluble mucoids from swollen sections	Phosphate	6.80	-7.8
	Acetate	4.7	-7.2
Soluble mucoids from water-extracted residues	Phosphate	6.82	-9.12
Aqueous extract	Phosphate	6.80	-8.05
Phenol-insoluble mucoid from acetone-dried sections	Phosphate	6.90	-9.0
	Acetate	3.97	-8.6
Phenol-insoluble mucoid from swollen sections	Phosphate	6.25	-9.2
Phenol-insoluble mucoid from water-extracted residues	Phosphate	6.82	-9.4
Trypsin product from dry sections	Phosphate	6.80	-9.25
Trypsin product from swollen sections	Phosphate	6.64	-9.4
Trypsin product from aqueous residues	Phosphate	6.62	-9.4

Extraction of the soluble mucoids with phenol

That polysaccharides can be separated from proteins by extraction with phenol was first shown by Morgan & Partridge (1941), for mucoids of bacterial origin. Phenol has subsequently been used to reduce the protein content of mucoids from connective tissues (Rogers, 1945; Partridge, 1948b). When the soluble mucoids from all three types of corneal sections were extracted with 92 % (w/v) phenol at room temperature, a separation was achieved into a fraction soluble in phenol and one insoluble in phenol.

Phenol-insoluble fraction

The insoluble fraction was suspended in water and dialysed, when it gave an opalescent solution. A solid was precipitated by the addition of 2 vol. of ethanol, redissolved in water and dried by sublimation. The solid was extracted again with phenol, dialysed, precipitated with ethanol, dialysed, and dried by sublimation. So prepared, the phenol-insoluble material had the composition:

From dry sections: 8.0 % N, 18.4 % hexosamine, 3.6 % ester-S.

From swollen sections: 8.3 % N, 19.0 % hexosamine.

From water-extracted sections: 8.7 % N, 21.0 % hexosamine.

The phenol-insoluble fraction from dry sections separated into two components on electrophoresis at pH's 6.8 and 3.97. The slow component represented 25 % of the total refraction, of which an unknown amount was due to the 'boundary anomalies'. The slow component was not identical with the phenol-soluble fraction of the soluble mucoids since it persisted through successive phenol extractions and ethanol precipitations. A sample

was isolated and found to contain about 5-10 % hexosamine. The phenol-insoluble material from the swollen sections contained a small amount of this slow component, but the phenol-insoluble material from the water-extracted sections contained less than 2 % of a slow-moving component.

The fast components of these phenol-insoluble mucoids had similar mobilities (see Table 4), and a Hexosamine/Nitrogen (H/N) ratio of 2.5-3.0. A 0.5 % solution of the fast component of the phenol-insoluble material from the dry sections had $\log \eta_{rel} = 0.28$.

The amino-acid composition of the phenol-insoluble mucoid from the water-extracted residues was determined by paper chromatography. The presence of glycine, alanine, proline, hydroxyproline, glutamic and aspartic acids, arginine, lysine, valine, serine and threonine, and an area corresponding to phenylalanine and the leucines could be demonstrated. These amino-acids are those shown to be present in collagen by Bowes & Kenten (1949).

The results described above refer to mucoids obtained by extraction with calcium chloride solutions. When the water-insoluble residues of the cornea were extracted with 10 % sodium chloride, pH 8, water-soluble mucoids were obtained with 1.9 % ester-S. This, at pH 6.8, had a fast component in the Tiselius apparatus which was responsible for

55 % of the total refraction. When the dry mucoids were extracted with 92 % phenol, the nitrogen content of the insoluble fraction could not be reduced below 8.0 %. It is hence unlikely that the association between the polysaccharide and the protein in the corneal extracts is due to a specific action of the Ca^{++} ion.

Phenol-soluble fractions

The first phenol extract of the soluble mucoids gave a precipitate on addition of 3 vol. of ethanol, but only a slight turbidity was obtained from the

similar to that of the phenol-insoluble fraction and it would appear that both are derived from corneal collagen.

Treatment of the phenol-insoluble material with chloroform and amyl alcohol

An attempt was made to reduce the protein content of the phenol-insoluble mucoid by the 'Sevag' technique. 0.3 % solutions of the phenol-insoluble material from dry sections were made in 0.25M-NaCl, pH 4; 0.25M-NaCl, pH 8; and 10 % CaCl_2 , pH 8, and each was shaken with a mixture of 4 vol. of chloroform and 1 vol. amyl alcohol. The interfacial precipitate from the successive shakings was collected, and

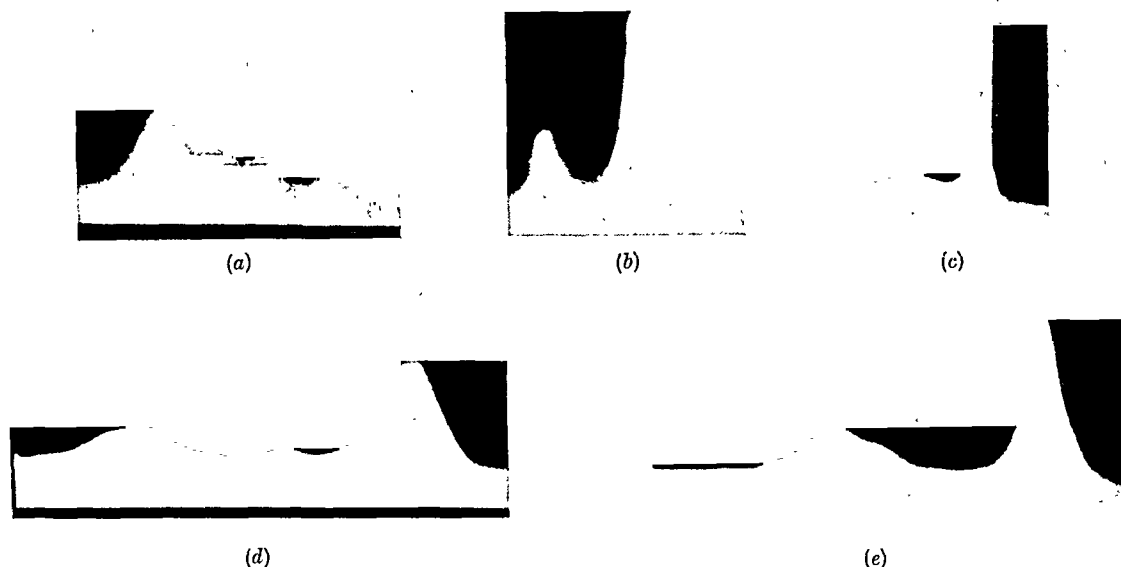


Fig. 3. Electrophoretic diagrams of corneal extracts. In all cases the buffer had the composition $I \text{ NaCl } 0.18$, $I \text{ buffer } 0.02$. The photographs have been taken with the slit at a high angle and are of the descending limbs with migration from left to right. (a) Aqueous extract, pH 6.8, 3 hr. from start. (b) Soluble mucoids from dry sections, pH 6.8, 2 hr. from start. (c) Phenol-insoluble mucoids from swollen sections, pH 6.8, 2.5 hr. from start. (d) Soluble mucoids from swollen sections, pH 6.8, 4 hr. from start. (e) Soluble mucoids from swollen sections, pH 4.7, 3 hr. from start.

second phenol extract. The solid precipitated from the phenol solution was dried and dissolved again in phenol. Dialysis of this solution against water gave a precipitate and a solution from which material was precipitated by the addition of 2 vol. of ethanol.

The solid produced by the addition of ethanol to the phenol extract of the soluble mucoids from the dry sections contained 0.25 % ester-S, 2.6 % hexosamine, 13 % nitrogen. The solid precipitated during the dialysis of the phenol extract of the soluble mucoid from the water-extracted sections contained 14.8 % nitrogen. The water-soluble, phenol-soluble, fraction of the same origin had a H/N ratio of 1.4.

The hydrolysate of the phenol-soluble, water-insoluble fraction derived from the water-insoluble residues of cornea had an amino-acid composition

washed with distilled water by centrifugation, till the supernatant was free from Cl^- . The precipitate contained 7.5–8.0 % N (not corrected for ash content). It appeared that the phenol-insoluble mucoid was precipitated unchanged.

Treatment of the mucoids with trypsin

Solutions of the phenol-insoluble material, and of the soluble mucoids, were adjusted to pH 8 and incubated at 37° with crystalline trypsin until the pH remained constant. About 10 mg. of trypsin were added for every 250 mg. of hexosamine present in the mucoids. A precipitate which appeared in the digest during the incubation was centrifuged off, and the supernatant dialysed. The N content of the soluble material could be reduced to 4–5 % by this process. The dialysed digest was diluted to give an 0.2 % solution in 10 % CaCl_2 , pH 8, and shaken with chloroform and amyl alcohol. When no further interfacial precipitate appeared, the process was repeated at pH 4 and the interfacial precipitate

removed. The solution was finally dialysed against 0.001 N-HCl, precipitated from the acid solution by 2 vol. ethanol, redissolved in water, dialysed, and dried by sublimation. After dialysis against 0.001 N-HCl and precipitation from the acid solution with ethanol, 10 mg. had no weighable ash so that analytical figures refer to the free acid and not to a salt.

The nitrogen content of the 'trypsin products' varied between 2.9% and 4.0%, and an electrophoretic examination revealed that they were not homogeneous. Preparations from all three types of corneal section had a fast-moving component and a material of very low mobility. In the trypsin product from the water-extracted sections, this slow component was responsible for 25% of the refraction, and in the other products, rather more. The fast component contained 3.3% nitrogen, 35% hexosamine, and 6.2% ester-S. The fast component of the trypsin product from the dry sections had $\log \eta_{rel.} = 0.14$ for a 0.5% solution.

Analysis by chromatography on filter paper

Analysis of mucopolysaccharides which contain uronic acids and hexosamines is complicated by the fact that the hydrolysis can proceed in stages with destruction of the more labile residues before all the glycosidic linkages are broken. Both degradation products and products of partial hydrolysis can be expected in the hydrolysate.

The analyses reported below were made on the fast component of the trypsin product, isolated after electrophoresis at pH 6.8. The same results have been obtained whatever the origin of the trypsin product. The hydrolysis conditions used have been N-sulphuric acid at 100° for 6 hr. and 5N-sulphuric acid at 100° for 6 hr. The polysaccharide yielded 78% reducing substances (as glucose) after the hydrolysis with 5N acid; with the N acid hydrolysate the reducing power was 38%. It

Table 5. *Hydrolytic action of hyaluronidase*

(All the corneal substrates were derived from acetone-dried sections. The liberation of less than 2.5% of the total reducing sugar is not significant and has been neglected in column 5.)

Substrate	Enzyme	Incubation time	Reducing sugar available (mg.)	Total reducing sugar liberated (%)
Hyaluronic acid	Streptococcal	1 hr.	1.18	24
	<i>Cl. welchii</i>	1 hr.	1.18	8
Phenol-insoluble mucoids	Streptococcal	1 hr.	1.48	0
	<i>Cl. welchii</i>	1 hr.	1.48	0
Hyaluronic acid	Streptococcal	49 min.	2.61	19
	Testis	49 min.	1.69	9
Fast component of phenol-insoluble mucoids	Streptococcal	6 hr.	2.05	0
	Testis	6 hr.	2.05	0
Trypsin product	Streptococcal	4 hr.	2.06	0
	Testis	4 hr.	2.06	0

Action of hyaluronidase on the mucoids and mucopolysaccharide

Table 5 gives the results of incubating the corneal extracts with hyaluronidase. The depolymerase activities of the enzyme preparations were such that 0.1 ml. of all enzymes tested disaggregated the hyaluronic acid in 2 ml. of the filtrate from ox vitreous body in less than 60 sec. That they possessed a hydrolytic activity is shown by the reducing sugars liberated from the hyaluronic acid preparation. The reducing sugar available in the hyaluronic acid preparation was calculated from the apparent hexosamine content; it is probable that hyaluronic acid is not the only substance containing hexosamine in this preparation (Pirie, 1949), and the figures given for the percentage of hydrolysis are probably too low. In calculating the percentage hydrolysis of both the hyaluronic acid and the corneal material, the assumption was made that reducing sugar equivalent to 2 equiv. of glucose was present for every equivalent of hexosamine.

was found that all the ester-S was removed by hydrolysis in N-hydrochloric acid for 6 hr. at 100°, and it is presumed that sulphuric acid has the same effect.

Chromatogram irrigated with collidine

After addition of the hydrolysate and the standard sugars to the paper, 5N-ammonia was added to each spot and allowed to dry off at room temperature. The paper was then irrigated for 48 hr. On development, the presence of galactose, glucosamine, and galactosamine in, and the absence of glucurone and glucuronic acid from, both hydrolysates could be demonstrated unambiguously. There was also present in the N-sulphuric acid hydrolysate, a substance which extended from the base line to a position 0.2 the distance moved by galactose, and which reacted with all three spraying reagents. It was absent from the hydrolysate treated with Zeo-Karb and reduced in intensity in that treated with De-Acidite. The 5N-sulphuric acid hydrolysate also contained some reducing material of low mobility

which gave a diffuse trail from the base-line to a position 0.3 the distance moved by galactose.

Chromatogram irrigated with phenol

Galactose and a hexosamine were present in the *N*-sulphuric acid hydrolysate, and also material of low mobility which after 36 hr. irrigation separated into two spots and reacted with all three spraying reagents. The slower spot moved 0.15 the distance moved by galactose and was absorbed by the Zeo-Karb but not by De-Acidite. The faster moved 0.35 the distance moved by galactose and was absorbed by both resins. No amino-acids could be detected in 0.5 mg. of polysaccharide hydrolysed in 5*N*-sulphuric acid.

a potential across the paper with 0.01*N*-ammonia as electrolyte. One of the slow-moving reducing substances moved to the anode.

No glucurone was present in the hydrolysate. Its absence is probably not due to destruction since a glucurone spot was found in a chromatogram from a mixture of galactose, glucurone and glucosamine, which had been put through the *N*-sulphuric acid-hydrolysis procedure.

Ionophoresis on filter paper

After the addition of the hydrolysate, or the standard sugars, to the papers 5*N*-ammonia was added to the spots and allowed to dry off at room temperature. This was a necessary procedure to

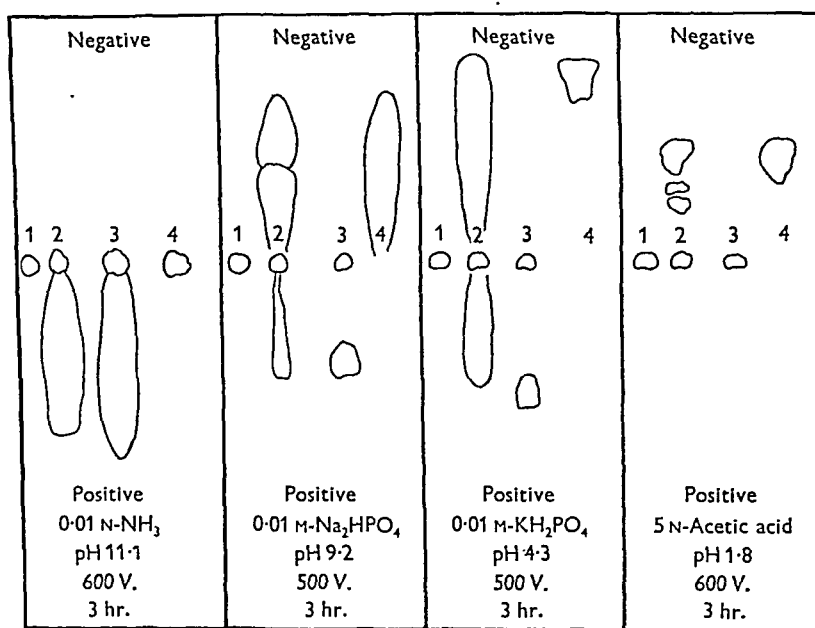


Fig. 4. Diagram of papers developed with aniline hydrogen phthalate after ionophoresis. 50 μ g. of the standard sugars and 300 μ g. of the hydrolysate were added to the spots. Positions are relative to galactose and not to the initial position. 1 = Galactose; 2 = *N*-H₂SO₄ hydrolysate; 3 = glucurone + NH₃; 4 = glucosamine hydrochloride.

Chromatogram irrigated with butanol-acetic acid

After 36 hr. irrigation, galactose and glucosamine were not separated. The *N*-acid hydrolysate showed in addition to these substances two reducing materials, the slower moving 0.35 as far as galactose, and the faster 0.59 as far as galactose. The slower was removed by both resins, the faster by the Zeo-Karb only. That the absorption by the De-Acidite was due to a negative charge on the molecule and not to an unspecific absorption was shown by first irrigating the chromatogram with butanol-acetic acid and then, after removal of the solvent, applying

ensure the conversion of some of the glucurone to ammonium glucuronate.

The results are summarized in Fig. 4. Glucosamine is not charged at pH 11 and glucuronic acid could not be detected in the glucurone at pH 1.8. It is clear that there is a negatively charged sugar ion in the polysaccharide hydrolysate. Acetic acid was the only solvent in which the hydrolysate gave discrete spots on ionophoresis. In the other solvents the charged species in the hydrolysate gave pear-shaped trails. The separation of the positively charged material into three spots with acetic acid as solvent is of interest; such an effect would be

expected if the hydrolysate contained partial hydrolysis products, or degradation products, in which the hexosamine was combined with neutral or weakly acidic substances.

Fractionation of the insoluble mucoids

The composition of the insoluble mucoids obtained from dry sections by calcium chloride extraction was 15% nitrogen, 3.5% hexosamine, 0.6% ester-S. The insoluble mucoids from sodium chloride extracts had a similar composition.

At least 0.5% sodium chloride was necessary to keep the insoluble mucoid in solution. As the solutions were very viscous, even in the presence of this concentration of salt, they were not analysed electrophoretically. The insoluble mucoids cannot be digested by trypsin.

The insoluble mucoids from the dry sections were dissolved in 10% CaCl_2 and the solution precipitated with 3 vol. of ethanol and a final concentration of 0.5% sodium acetate. The precipitate contained 13.5% N, and 6.0% hexosamine. The dry solid was extracted with 92% phenol solution at room temperature. The insoluble fraction was suspended in water and dialysed, when it dissolved to give an opalescent solution, from which the addition of 2 vol. of ethanol precipitated a white solid.

The ethanol precipitate contained 9% nitrogen, 17% hexosamine, and contained ester-S. A hydrolysate made in N-sulphuric acid showed glucosamine and galactose but no glucuronic acid. Moreover, this hydrolysate contained the slow-moving reducing material present in the N-sulphuric acid hydrolysate from the trypsin product.

However, the chromatograms were not so clear as those from the trypsin product and it was not possible to demonstrate the presence of galactosamine conclusively.

Phenol-soluble fraction of the insoluble mucoids

The phenol extract of the insoluble mucoids was dialysed against water and the precipitate collected; the suspension in water was frozen and the water sublimed off; all the solid redissolved in phenol and was precipitated by 3 vol. of ethanol. This solid contained 16.8% nitrogen.

It appeared from some of its properties that the protein component of the insoluble mucoids was derived from a collagen. It was thought that a test of this origin of the protein would be its further degradation to gelatin. Accordingly, the solid precipitated, by dialysis, from the phenol extracts of the insoluble mucoid was suspended in distilled water (pH 5.5) and heated at 105° for 4 hr. All but a small fraction dissolved to give a clear solution, which set to a gel on cooling, when the concentration was greater than about 1.5%.

This protein was examined in the Tiselius apparatus. To enable comparison with the mobilities

of gelatin derived from the collagen in cartilage, the buffer solutions were the same as those used by Partridge (1948b). As the mobilities were low, results of the highest accuracy would require a buffer of smaller ionic strength. The protein was not homogeneous (Fig. 5). The difference in the mobility of the components was small, but was most marked in acetate buffers in the range pH 3–5. Table 4 gives the mobilities of the fast component; on the acid side of the isoelectric point they differ slightly from those for gelatin from cartilage.



Fig. 5. Electrophoresis diagram of the gel-forming protein from the insoluble mucoids. The photograph is of the ascending limb, with migration from right to left. Acetate-NaCl buffer, pH 3.74. 7 hr. from start, current 0.01 amp.

Table 6. *Electrophoretic mobilities of the gel-forming protein from the insoluble mucoid*

(The buffer contained NaCl ($I_{\text{NaCl}}=0.18$, $I_{\text{buffer}}=0.02$). The mobilities are the means of the values from each limb. Where the boundaries split into two peaks the mobility refers to the fast component.)

Buffer	pH	Mobility ($\mu \times 10^5$)
Phosphate	6.80	-1.0
Phosphate	6.80	-1.1
Phosphate	5.34	-0.85
Acetate	4.92	-0.52
Acetate	3.95	+1.28
Acetate	3.74	+1.44
Glycine	2.75	+2.95
Glycine	2.70	+2.65

Paper chromatography of a hydrolysate from the gel-forming proteins from cornea showed that the amino-acid composition was very similar to that of gelatin.

DISCUSSION

About 70% of the acid mucopolysaccharide has been extracted in an electrophoretically homogeneous form. The residues remaining after extraction of the swollen sections contain about 15% of the total ester-S and nothing is known about this fraction. The insoluble mucoids contain about 10–15% of the total ester-S and there is not sufficient evidence to permit identification of the carbohydrate components of the two mucoids. The

only evidence for the homogeneity of the mucopolysaccharide is the electrophoretic measurements. The chemical fractionation was designed to remove protein and the persistence of the mucopolysaccharide through this is not evidence for its homogeneity.

The identification of galactose as a constituent of the corneal mucopolysaccharide is of interest. Suzuki (1939) considered that galactose was present but, as he used an unspecific method of identification, Meyer & Chaffee (1940) considered that he had confused galactose and a uronic acid. I have not found a uronic acid as a constituent of the N-acid hydrolysate of the mucopolysaccharide. However, the lability of the uronic acids and the resistance of hexosaminosides to acid hydrolysis is well known, and it is possible that its absence is due to both destruction and insufficient hydrolysis. Moreover, one of the partial hydrolysis products has an acid group and that may be due to the presence of combined uronic acid. It is probable that the identification of the uronic acid stated to be present in the corneal mucopolysaccharide will be best achieved by protection of the labile groups of the molecule before hydrolysis.

It appears from the results of Kaye & Stacey (1951) that galactose is not a constituent of hyaluronic acid, and accordingly the suggestion of Meyer & Chaffee (1940) that the corneal mucopolysaccharide is a sulphate ester of hyaluronic acid is invalidated by the results described in the present paper.

The mucopolysaccharide is not hydrolysed by hyaluronidase preparations. The failure of the enzyme to have a spreading action in the cornea has already been reported (Woodin, 1950*a*), and it was suggested in that paper that an explanation of the failure to confirm the results of Meyer & Chaffee would be that the pneumococcal hyaluronidase used by these authors contained enzymes other than hyaluronidase. Pneumococcal infection produces a characteristic spreading ulcer in the cornea and presumably the spread is effected by enzymic processes.

The extraction experiments were designed primarily to determine the optimum conditions to bring the mucoid into solution in an undegraded form. Although a knowledge of the effect on the cornea of calcium chloride and sodium chloride in varying concentrations will be required before an explanation of these results can be offered, it is of interest that the effect of sodium chloride and calcium chloride in extracting the mucopolysaccharide from the cornea should parallel the effects of these substances in reducing the shrinkage temperature, and increasing the swelling pressure of the collagen in rat-tail tendon and in sheep skin respectively. Bowes & Kenten (1950) have suggested that the decrease in the cohesion brought

about by calcium chloride is primarily due to a reduction in the potential energy of the inter-molecular links between the polypeptide chains. The results presented here suggest that the cohesive forces of the collagen fibres and the forces holding the mucopolysaccharide to the corneal collagen are very similar. It would be of great interest to know if the mucopolysaccharide of the cornea were contributing to the cohesion of the corneal collagen, since the orderly arrangement of the collagen fibres is probably a necessary condition of the transparency of the tissue.

The corneal mucopolysaccharide can form stable complexes with proteins. The fast component of the phenol-insoluble mucoids, which contains about 40 % protein, is homogeneous electrophoretically in the range pH 8-4 and is resistant to chemical fractionation by the 'Sevag' technique and by extraction with phenol. The fast component of the soluble mucoids from the water-extracted residues, and the phenol-insoluble material, have the same mobility and H/N ratio and they are probably identical. However, the fast component of the soluble mucoids from dry sections and from the swollen sections contains more protein than this complex; it is homogeneous in the range pH 9.05-4.7, and chemical combination can be presumed to occur between the protein and mucopolysaccharide components. The mobility of this fast component is smaller than that of the phenol-insoluble material, and it is at least possible that this soluble mucoid contains a complex formed by polar association between the phenol-insoluble material and some of the water-soluble corneal proteins.

It is of interest that the proteins with which the mucopolysaccharide is associated have an amino-acid composition qualitatively the same as that of gelatin. This fact, considered in conjunction with the results of the extraction experiments, suggests that the unit in the intact stroma is a mucopolysaccharide-collagen complex.

SUMMARY

1. The ester-S present in the cornea corresponds to a mucopolysaccharide content of 4.2 %. There is an excess of hexosamine over that required as a constituent of the mucopolysaccharide.

2. By extraction of the water-swollen sections of cornea with 10 % calcium chloride at pH 8, 85 % of the mucopolysaccharide can be dissolved. Extraction with calcium chloride is more efficient than extraction with sodium chloride and that is more efficient than extraction with water.

3. The mucopolysaccharide is extracted as a component of both water-soluble and water-insoluble mucoids.

4. The protein content of the water-soluble mucoids can be reduced by extraction with phenol

to leave a phenol-insoluble mucoid. This contains a substance with the characteristics of a mucopolysaccharide-protein complex.

5. The water-soluble mucoids contain a complex which is formed by interaction between the phenol-insoluble mucoids and a water-soluble protein of the cornea.

6. The acid mucopolysaccharide can be prepared free from protein by incubation with trypsin. Galactose, glucosamine and galactosamine have been identified as constituents of the mucopolysaccharide, by chromatography and ionophoresis on filter paper.

7. The corneal mucopolysaccharide is not a substrate for hyaluronidase.

8. Gel-forming proteins have been isolated from the insoluble mucoid and found to contain the same amino-acids as gelatin, but to differ from it in their electrophoretic behaviour.

9. It is suggested that *in situ* the acid mucopolysaccharide is combined with proteins of the collagen group.

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The Synthesis of Cozymase from Nicotinic Acid and its Derivatives by *Lactobacillus arabinosus* 17-5

By D. E. HUGHES AND D. H. WILLIAMSON

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
The University, Sheffield 10

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As shown in previous papers from this laboratory (McIlwain, 1949; McIlwain, Stanley & Hughes, 1949), glycolysis by washed suspensions of *Lactobacillus arabinosus* 17-5, which have been grown on a nicotinic acid-deficient medium, is stimulated by the addition of nicotinic acid. The stimulation is due to the synthesis of cozymase and, since this organism does not rapidly decompose the coenzyme (McIlwain & Hughes, 1948), it seemed to be suitable material in which to study its synthesis. In the present studies, the effect of limiting amounts of nicotinic acid, nicotinamide and nicotinamide nucleotides on the stimulation of glycolysis and on the synthesis of

cozymase has been investigated. Young deficient cells glycolysing in an optimal concentration of nicotinic acid rapidly synthesized cozymase until the cells became saturated with the coenzyme; synthesis of cozymase and metabolism of nicotinic acid then ceased. Glucose alone of the constituents of the growth medium was essential for the synthesis of cozymase by washed suspensions. The uptake of nicotinic acid ran parallel to the synthesis of coenzyme and no detectable amounts of any possible intermediate in the synthesis were detected in cells or the suspending fluid. Unexpectedly, nicotinamide was found to be rapidly deamidated by the

washed suspensions; deamidation was independent of the glucose and of the cozymase content of the cells.

EXPERIMENTAL

Growth conditions for obtaining cozymase-deficient cells

Lactobacillus arabinosus 17-5 was grown as described by McIlwain & Hughes (1948) on the semi-synthetic medium consisting of casein hydrolysate, salts and vitamin supple-

washed out twice with 1.0 ml. 0.9% NaCl, the washings were added to the centrifuged cells and recentrifuged. The washings were discarded and the cells washed once again with 2.0 ml. of 0.9% saline, and then resuspended in 1.0 ml. of 0.065M-phosphate buffer, pH 6.2, and heated at 80° for 15 min. As shown previously (McIlwain & Hughes, 1948) this treatment releases the cozymase from the cells with a 5-8% loss; no corrections for this loss were made. After heating, the tubes were cooled in running tap water and if not used immediately the contents were frozen solid and stored at -14°.

Table 1. *The effect of nicotinic acid upon glycolysis by washed suspensions of Lactobacillus arabinosus* 17-5

(The cells were grown as indicated below, collected, washed as described and the effect of nicotinic acid on glycolysis measured manometrically. In each Warburg flask was 2.0 ml. containing: 0.05M-NaHCO₃; 0.05M-glucose; 2.0-4.0 mg. dry wt. cells. Nicotinic acid (5×10^{-5} M final) was added from the side arm.)

Growth period (hr.)	Nicotinic acid in growth medium (M $\times 10^{-7}$)	Yield of cells (mg. dry wt./100 ml.)	CO ₂ (μ moles/mg. dry wt./hr.)	
			Without nicotinic acid	With nicotinic acid
19	100	30	11.0	11.0
19	10	30	10.0	10.0
60	10	45	3.7	6.0
22	5.0	12	17.0	17.0
40	5.0	24	3.0	10.0
21	2.0	15	7.2	11.0
40	2.0	16	3.6	10.8
22	1.0	9	12.0	20.0
40	1.0	10	5.0	15.0
20	0.4	35	10.0	20.0

ment. Satisfactory yields (10-15 mg. dry wt./100 ml.) of deficient cells were obtained after 30-44 hr. growth on the medium containing from 1.5 to 2.0×10^{-7} M-nicotinic acid (Table 1). The addition of nicotinic acid to glycolysing suspensions of these deficient cells stimulated their rate of glycolysis from two- to three-fold. Higher yields of deficient cells (30-40 mg. dry wt./100 ml.) were obtained after 60-70 hr. growth on the medium containing more nicotinic acid (10^{-6} M), but these cells were unsatisfactory because their rate of glycolysis tended to fall on incubation and the ability to synthesize cozymase was poor. Non-deficient cells were grown on a medium made from enzymically hydrolysed casein, yeast extract and glucose (Nossal, 1951).

Measurements of the effects of nicotinic acid on glycolysis and cozymase synthesis

In the main compartment of a conical Warburg cup was placed 0.1M-glucose, 0.5 ml.; 0.2M-NaHCO₃, 0.5 ml.; washed cell suspension, 0.5 ml. and sufficient Krebs & Henseleit saline to bring the final volume to 2.0 ml. The nicotinic acid or derivative was in the side arm in 0.2 ml. water. Yellow phosphorus was placed in the centre well. The cup was gassed with 95% N₂-5% CO₂, and shaken in a thermostat at 40.0°. Readings were taken at 5 min. intervals until the rate of evolution of CO₂ was constant (20-30 min.). The side arm was then emptied and readings continued at 3-5 min. intervals. To stop the reaction, the cup was taken from the thermostat and placed in an ice-salt freezing mixture. The contents were then allowed to thaw, transferred to a centrifuge tube and centrifuged for 10 min. at approx. 3500 g. The supernatant was poured off into a test tube. In order to collect the cells quantitatively the cup was

Determination of cozymase

Cozymase was determined manometrically in the apozymase system of Axelrod & Elvehjem (1942), essentially as described by McIlwain & Hughes (1948). The apparent loss of activity by many batches of apozymase upon storing was found to be associated with the formation of acidic material at 2°. This lowered the pH of the apozymase system from 6.2 to 5.6-5.8 and thus reduced its activity. In later experiments where the apozymase was dried over P₂O₅ and stored over a drying agent such as silica gel at -14° the apozymase lost no activity in a month and lost from 10 to 15% after 2 months. Large blank values in some experiments were found to be due to cozymase present in the calcium hexosediphosphate (obtained from Schwartz Laboratories Inc., New York 17, U.S.A.). To remove the cozymase, the calcium hexosediphosphate was dissolved in 1.1 equivalents of HCl and stirred with one-tenth of its weight in norite for 0.5 hr. at 2°. The norite was removed by filtration and 2 equivalents of Ba(OH)₂ added. The pH was adjusted to 8.5 and the precipitate of dibarium salt of hexosediphosphate was filtered off, washed with water and ethanol and dried. Barium was removed from small samples immediately before use by the addition of an equivalent of 0.5N-Na₂SO₄ to an ice-cold solution of the barium salt in 2N-HCl.

Determination of nicotinic acid, nicotinamide and nicotinamide derivatives

In most of the experiments, nicotinic acid was determined by the microbiological assay method of Barton-Wright (1946). When it was not desired to distinguish between nicotinic acid and amide, samples for assay were

heated in N -HCl as described in a previous paper (McIlwain & Hughes, 1948). To distinguish between the acid and amide, a portion of the sample was treated with sodium hypobromite as described by Atkin, Schultz, Williams & Freys (1943), to destroy the nicotinamide, and another portion was heated with HCl as before. The nicotinic acid in both portions was estimated by microbiological assay and the nicotinamide content of the sample calculated by difference. In some of the later work, nicotinic acid and nicotinamide were estimated by the chemical methods of Chaudhuri & Kodicek (1948) and Hughes (1949). The acetone condensation method of Levitis, Robinson, Rosen, Huff & Perlzweig (1945) was used to determine the total concentration of N^1 -substituted nicotinamide derivatives. Fluorescence was estimated in a fluorimeter employing a photomultiplier tube and similar to that described by Lowry (1948). Solutions of quinine sulphate were used as permanent standards.

Cozymase, nicotinamide ribotide and riboside can act as 'V' factor for bacteria (Gingrich & Schlenk, 1944). Activity as 'V' factor was estimated by measuring the growth response of *Haemophilus parainfluenzae* as described by McIlwain & Hughes (1948). National Type Culture Collection, strains no. 4101, no. 4625 and eight locally isolated strains were tested. Where the strain was found to reduce nitrate, tests for nitrite were made according to Hoagland & Ward (1942). It was found that although nitrite was often detected where no growth could be seen, the response of various strains towards nitrate was too unreliable to be used as a quantitative method for estimating 'V' factor.

Chemical determinations

Ammonia was distilled in a modified Parnas apparatus or in Conway units and determined by the method of Russell (1945) or with Nessler's reagent. A modification of the method of Van Slyke was used for the estimation of glucose (see Wheatley, 1947). The ribose and phosphate contents of the nicotinamide nucleotides were determined as described by Schlenk (1943). Lactic acid was estimated by the method of Friedman & Graeser (1933).

Materials

The first of the two specimens of cozymase used as a standard was that described as 'C' by McIlwain & Hughes (1948); it contained 44% coenzyme I (CoI) and no detectable free nicotinic acid or nicotinamide. The second specimen was prepared from brewer's yeast according to LePage (1949), and contained 50–52% CoI when compared with the first specimen in the apozymase system, and approximately 2.0% free nicotinamide, as judged by microbiological assay. Specimens of dihydronicotinamide ribofuranoside (nicotinamide riboside) were made available through the courtesy of Dr L. J. Haynes, Chemistry Department, University of Cambridge. Tests for 'V' factor activity showed that it had one-quarter to one-tenth of the activity of CoI on a molar basis as 'V' factor (Table 2) (see also Haynes & Todd, 1950). In most freshly isolated strains the amounts of riboside needed for visible growth were higher than those needed in the older strains (see Schlenk & Gingrich, 1944). Specimens of nicotinamide glucoside, galactoside and arabinoside were also synthesized by Dr Haynes; they were without 'V'-factor activity for all strains tested (Table 2). Nicotinamide ribose phosphate (nicotinamide ribotide) was

prepared from cozymase by the action of potato pyrophosphatase according to Kornberg & Pricer (1949). Nicotinic acid and nicotinamide were commercial specimens which had been recrystallized several times from water-ethanol mixtures.

Table 2. 'V'-factor activity of nicotinamide nucleosides

(*Haemophilus parainfluenzae* Strain 4101 was grown as described by McIlwain & Hughes (1948) and growth measured photometrically. Nitrite was detected by the method of Hoagland & Ward (1942); + indicating just detectable amounts and + + + maximum amounts, and 0 no test for nitrite.)

Growth factor added	Concn. of nicotinamide derivative ($M \times 10^{-7}$)	Photo-meter reading at 22 hr.	Nitrite at 22 hr.
None	—	5.2	0
Cozymase	1.3	25	+ + + +
	0.75	20	+ + +
	0.35	13	.
	0.17	6	0
Nicotinamide riboside	20	20	+ + + +
	4	9	+ +
	1	5	+
	0.2	5	+
Nicotinamide galactoside	100	5	0
	10	5	0
Nicotinamide galactoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ +
	10	20	+ +
Nicotinamide arabinoside	100	5	0
	10	5	0
Nicotinamide arabinoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ + + +
	10	20	+ +
Nicotinamide glucoside	100	5	0
	10	5	0
Nicotinamide glucoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ + +
	10	20	+ +

RESULTS

Stimulation of glycolysis by varying concentrations of nicotinic acid

The addition of nicotinic acid in concentrations from 3×10^{-8} to $10^{-4} M$ to glycolysing washed suspensions of deficient cells caused a gradual increase in the rate of glycolysis (Fig. 1). The magnitude of the increase was greatest at and above 1.0 – $5.0 \times 10^{-6} M$; below this concentration the effect decreased and generally could not be detected in concentrations below $3 \times 10^{-8} M$ (Table 3). The effect reached its maximum after a lag period of 10–50 min. This lag period was shortest at nicotinic acid concentrations above $5 \times 10^{-5} M$, and increased as the concentration was reduced to $10^{-7} M$ (Fig. 1). This stimulating effect of nicotinic acid is due to the formation of cozymase which in the deficient cells limits glycolysis (McIlwain *et al.* 1949). Thus the lag period may

be taken as indicating the time taken for the cells to synthesize sufficient cozymase to saturate the glycolytic system and hence indirectly indicates the rate of cozymase synthesis. It would therefore appear that below $5 \times 10^{-5} M$ the concentration of nicotinic acid is a limiting factor in cozymase synthesis. When limiting amounts of nicotinic acid

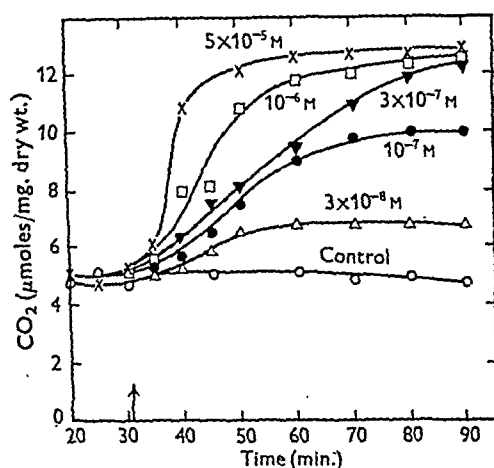


Fig. 1. The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells. Cells were grown for 41 hr. on medium containing $1.57 \times 10^{-7} M$ -nicotinic acid and collected and washed as described. Glycolysis was followed manometrically. In each Warburg cup were 2 ml. containing 0.05M-glucose, 0.05M- NaHCO_3 , 3.2 mg. dry wt. of cells. Nicotinic acid added from the side arm as indicated by the arrow.

Table 3. The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells

(Cells were grown for 40 hr. on a medium containing $1.5 \times 10^{-7} M$ -nicotinic acid. Glycolysis was followed manometrically. In each Warburg flask was 2.0 ml. containing: 0.05M-glucose; 0.05M- NaHCO_3 ; 2.3 mg. dry wt. of cells: the nicotinic acid was added from the side arm. The values for CO_2 production are the maximum that occurred during 2 hr. after adding the nicotinic acid.)

Concn. of nicotinic acid ($M \times 10^{-8}$)	CO_2 ($\mu\text{moles/mg.}$ dry wt. cells/hr.)
None	7.2
3.0	7.8
10.0	9.7
30.0	17.7
100	18.0
150	19.3
500	19.0

are added to the cells, all the nicotinic acid is taken up and converted into cozymase (see p. 335). A limiting concentration of 1.0 to $5.0 \times 10^{-6} M$ would mean therefore that 1.0–2.0 μmoles of nicotinic acid/mg. dry wt. are needed to saturate the glycolytic system of the cells.

The rate of cozymase synthesis at varying concentrations of nicotinic acid

Varying concentrations of nicotinic acid ranging from 5×10^{-4} to $3 \times 10^{-7} M$ were added to glycolysing deficient cells. The cups were placed in a freezing

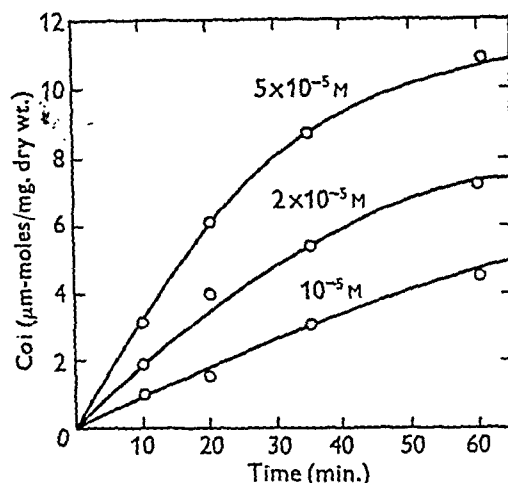


Fig. 2. Cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing $2 \times 10^{-7} M$ -nicotinic acid, collected and washed. Reaction with nicotinic acid, in concentrations as indicated on the graphs, and cozymase determinations were as described in the text.

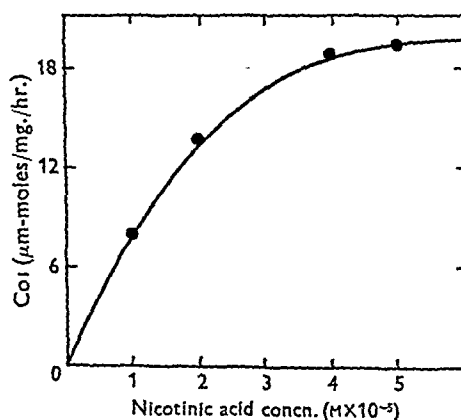


Fig. 3. The initial rate of cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing $2 \times 10^{-7} M$ -nicotinic acid. The reaction with nicotinic acid was for 10 min. at 40° . Cozymase estimations were carried out as described in the text.

mixture to stop the reaction at 5, 10, 15 and 30 min., the cells collected, cozymase extracted, and estimated as already described. Under these conditions no measurable amounts of cozymase appeared in the reaction fluid and it is therefore assumed that the rate of cozymase accumulation in the cells indicates

the rate of cozymase synthesis. As will be seen from Fig. 2 the cozymase content of the cells rapidly rises following the addition of nicotinic acid. At the higher concentrations of nicotinic acid, the maximum rate of cozymase synthesis lasted for approximately 20–30 min. then decreased and ceased when the cells contained 12–14 $\mu\text{m-moles/mg. dry wt.}$ (Fig. 2). At the lower concentrations of nicotinic acid the rate of cozymase synthesis continued approximately linearly until all the available nicotinic acid was converted into the coenzyme (see also p. 335). The maximum rate of cozymase synthesis (16–25 $\mu\text{m-moles/mg. dry wt./hr.}$) occurred in concentrations at and above $5 \times 10^{-5}\text{M}$ -nicotinic acid and fell off progressively as nicotinic acid concentration was reduced (Fig. 3). These results confirm the conclusions drawn from the experiments in the preceding paragraph, which suggested that the rate of cozymase synthesis was limited by the concentration of nicotinic acid.

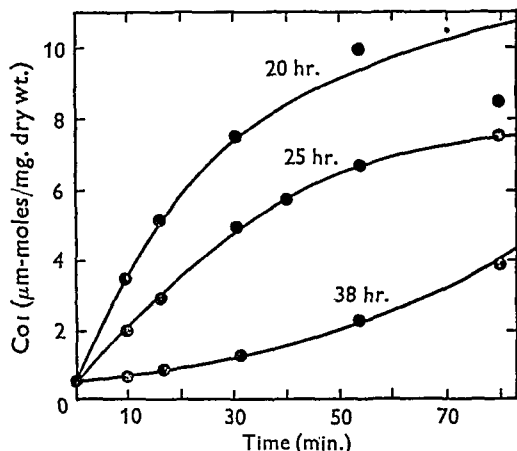


Fig. 4. The effect of growth period on the rate of cozymase synthesis. Cells were grown in medium containing $2 \times 10^{-7}\text{M}$ -nicotinic acid and samples taken at the times indicated. The cells were collected, washed and the reaction with nicotinic acid ($5 \times 10^{-5}\text{M}$ final) and cozymase estimations carried out as described.

The effect of growth period upon the cozymase synthesis

The maximum rate of cozymase synthesis, at and above $5 \times 10^{-5}\text{M}$ -nicotinic acid, was found in washed cells grown for 16–20 hr. In three batches of cells grown for a shorter period (10 hr.) the rate of synthesis was 15–20 % lower than maximum. The rate of cozymase synthesis fell progressively as growth was extended from 20 to 70 hr. (Fig. 4). At 38 hr. the initial rate was 1.5–2.0 $\mu\text{m-moles/mg. dry wt./hr.}$ and increased to 6.0–10.0 $\mu\text{m-moles/mg. dry wt./hr.}$ after 50–90 min. In cells grown for 70 hr., however, the rate of cozymase synthesis remained low (0.5–1.0 $\mu\text{m-mole/mg. dry wt./hr.}$) on incubation up to 3 hr. During this period the rate of

glycolysis increased from 1 $\mu\text{mole/mg. carbon dioxide/mg. dry wt./hr.}$ to 4 $\mu\text{moles/mg. dry wt./hr.}$ Younger cells with an initial rate of glycolysis of from 2 to 5 $\mu\text{moles carbon dioxide/mg. dry wt./hr.}$ were found to synthesize cozymase at rates of 2–4 $\mu\text{m-moles/mg. dry wt./hr.}$, that is at a rate two- to three-fold faster than in the old cells whose rate of glycolysis was the same. Although, as is shown later, glycolysis is essential for cozymase synthesis, it would appear that the synthesis in older cells is limited by processes other than glycolysis.

The effect of replacing nicotinic acid by nicotinamide and its derivatives

In young deficient cells (19–22 hr. growth) there was no significant difference in the effects on glycolysis or on the rate of cozymase synthesis when nicotinic acid was replaced by nicotinamide, nicotinamide riboside or nicotinamide ribotide. In older cells (30–48 hr. growth) glycolysis was stimulated more rapidly by the nicotinamide and its

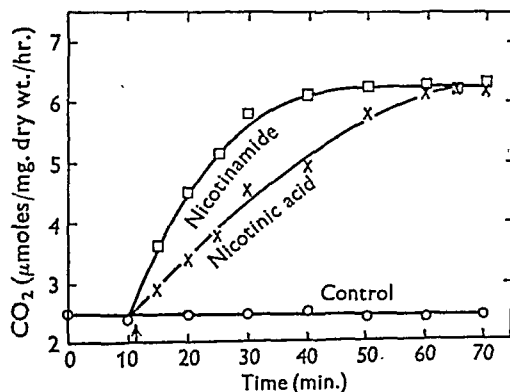


Fig. 5. Stimulation of glycolysis in deficient cells grown for 50 hr., by nicotinic acid and nicotinamide. Glycolysis and reaction with nicotinic acid and nicotinamide ($5 \times 10^{-5}\text{M}$ final) were measured manometrically as described in the text.

derivatives than by nicotinic acid, although the final rate of glycolysis was the same (Fig. 5). This suggests that in older cells cozymase is synthesized more rapidly from nicotinamide and its derivatives than from nicotinic acid. Experiments in which the cozymase content of the cells was measured showed no differences in the rate of cozymase synthesis. As shown previously the differences in the amount of cozymase which changed the rate of glycolysis were of the order of 1.0–2.0 $\mu\text{m-moles/mg. dry wt.}$ Differences in the rate of formation of such small amounts of cozymase were too small to be measured by the present methods. No cozymase was synthesized from nicotinamide arabinoside, nicotinamide glucoside or nicotinamide galactoside. This

result agrees with the finding that these compounds are inactive as 'V' factor for *Haem. parainfluenzae*.

Under a variety of conditions the rate of cozymase accumulation in deficient cells was, in the presence of cozymase, 10-15 % faster than the accumulation in the presence of nicotinic acid or nicotinamide. Cozymase was not degraded by the organism at rates faster than 0.01 μ m-mole/mg. dry wt./hr. (see also McIlwain & Hughes, 1948). The intact cozymase molecule thus appears to be readily adsorbed.

and no nicotinamide or other possible intermediates in the synthesis of cozymase accumulated once the saturation level was attained (Table 5). When experiments were continued for longer periods, i.e. up to 4 hr. instead of the usual 1-2 hr., a small amount of an N^1 -substituted nicotinamide compound was detected in the medium. This material had 'V'-factor activity for *Haem. parainfluenzae*, but the amounts as judged by the fluorimetric method of Levitis *et al.* (1945) were equivalent to 0.02 μ m-mole

Table 4. Cellular concentration of nicotinic acid, nicotinamide or cozymase during synthesis by deficient cells

(Cells were grown on medium as described below, collected and washed. The reaction with nicotinic acid ($5 \times 10^{-5}M$) and estimation of the products was carried out as described in the text.)

	Time after adding nicotinic acid (min.)	Total nicotinic acid (μ moles/mg. dry wt.)	Nicotinamide (μ moles/mg. dry wt.)	Cozymase (μ moles/mg. dry wt.)
Exp. A. Cells were grown for 28 hr. on medium containing $2 \times 10^{-7}M$ -nicotinic acid	0	0.5	—	0.46
	10	3.6	—	3.8
	20	6.3	—	6.3
	40	11.1	—	10.8
	80	15.6	—	14.0
Exp. B. Cells were grown for 44 hr. on medium containing $1.57 \times 10^{-7}M$ -nicotinic acid	0	1.2	1.0	1.0
	30	1.8	1.2	1.4
	60	5.0	3.0	3.2

Cozymase was not taken up in the absence of glucose; some source of energy is therefore needed for cozymase adsorption as well as for its synthesis. The highest concentration of cozymase found in cells was the same (10-12 μ m-moles/mg. dry wt.) whether nicotinic acid, nicotinamide or cozymase were added to glycolysing deficient cells. Cells grown with adequate nicotinic acid ($10^{-4}M$) on the semi-synthetic medium or on the yeast-supplemented pronutrin medium also contained the same amount of cozymase and did not adsorb further cozymase. This amount of cozymase (10-12 μ m-moles/mg. dry wt.) therefore appears to be a saturation level for this organism and once it is attained the organism ceases either to adsorb further cozymase from the medium or to destroy it as in the case of other micro-organisms (McIlwain & Hughes, 1948).

The metabolism of nicotinic acid

In the absence of glucose, nicotinic acid was not removed from the medium by washed suspensions of cells which had been grown under a variety of conditions. In the presence of glucose, however, nicotinic acid was taken up by deficient cells at approximately the same rate as cozymase was synthesized (Table 4). During synthesis, small amounts of free nicotinic acid sometimes appeared in the cells, but when the cozymase content of the cells had reached 10-12 μ m-moles/mg. dry wt. cozymase synthesis and nicotinic acid metabolism ceased. When the cells were provided with up to tenfold excess of nicotinic acid the excess acid was recovered from the medium

of cozymase/2.0 ml. and were too small to be estimated in the apozymase system. It is uncertain, therefore, whether the substance leaking from the cells is cozymase or some other N^1 -substituted nicotinamide derivative.

Table 5. Recovery of nicotinic acid from medium and cells after glycolysis

(Nicotinic acid was added to glycolysing cells (3.0 mg./2 ml.) under the conditions described in Tables 2 and 3. After reacting for 90 min. the cells were collected by centrifugation, washed twice with 0.9 % NaCl, and nicotinic acid was estimated in cells and medium by microbiological assay.)

Nicotinic acid added to the medium (μ moles)	Nicotinic acid recovered (μ moles)	
	Cells	Medium
None	2.0	None
6.0	8.0	None
20	16	1.5
50	19	3.1
100	23	80
200	25	180

The metabolism of nicotinamide

Nicotinamide added to glycolysing cells disappeared rapidly from the medium as judged by the fluorescence after reaction with CNBr (Chaudhuri & Kodicek, 1948) (Table 6). Some of the nicotinamide (20-40 μ moles) which disappeared from the medium was accounted for by the appearance in the cells of cozymase, but the rest could not be accounted

for by the accumulation of other derivatives of nicotinic acid or nicotinamide (Table 6). After reaction with nicotinamide the mother liquor was examined by the method of Hughes (1949) (reaction with CNBr at pH 5.5, followed by sodium hydroxide), and the absorption spectrum was found to

since blank values on the cells themselves were sometimes as large as that expected to be formed from the nicotinamide (100 μ m-moles). However, ammonia formation at the rate of approximately 2 μ moles/mg. dry wt./hr. was found when cells were incubated with larger amounts of nicotinamide than

Table 6. *Deamination of nicotinamide during cozymase synthesis*

(The reaction was carried out as described in the text. In each Warburg flask were 2.0 ml. containing: 0.05M-glucose; 0.05M-NaHCO₃; 2.3 mg. dry wt. cells. Nicotinamide (100 μ m-moles) was added from the side arm.)

Time after adding nicotinamide (min.)	Relative fluorescence	Nicotinic acid in mother liquor (μ m-moles/2.0 ml.)	NH ₃ content of mother liquor (μ m-moles/2.0 ml.)	Cozymase content of cells (μ m-moles)
0	100	0	57	1.2
8	2.7	71	75	8.2
15	*Negative reading	84	87	12.0
30	*Negative reading	85	54	19.0
60	*Negative reading	80	50	19.3

* Negative reading indicates some absorption of the activating light probably by nicotinic acid (cf. Chaudhuri & Kodicek, 1948).

resemble that of nicotinic acid (Fig. 6). The formation of nicotinic acid from nicotinamide was confirmed by microbiological assay (Table 6). It was also found that there was some temporary increase

usual (2.0×10^{-3} M). A similar formation of ammonia occurred in the absence of glucose (Table 7). It is clear, therefore, that these organisms deaminate nicotinamide and that this reaction, unlike the uptake of nicotinic acid and the synthesis of cozymase, is independent of the presence of glucose.

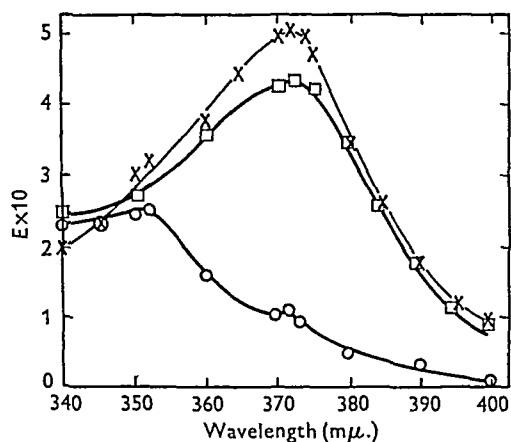


Fig. 6. The change in the absorption curve of nicotinamide brought about by *Lb. arabinosus*. Nicotinamide (100 μ m-moles/2 ml.) was added to glycolysing cells under the usual conditions and the reaction stopped at 0 and 10 min. The cells were removed by centrifugation, 1.0 ml. of the mother liquors reacted with CNBr and NaOH and the absorption spectra measured in a Beckman spectrophotometer. O—O, solution at 0 min. The absorption spectra is typical of nicotinamide (E_{\max} at 352 $m\mu$). □—□, solution at 10 min. (E_{\max} at 372 $m\mu$). x—x, control solution of nicotinic acid (E_{\max} at 373 $m\mu$).

in the ammonium content of the medium during reaction with nicotinamide by glycolysing cells under these conditions (Table 6). The amount of ammonia expected to be formed under the usual conditions was too small to be accurately estimated

Table 7. *Nicotinic acid and NH₃ formation from nicotinamide in the absence of glucose*

(*Lb. arabinosus* was grown on the Pronutrin medium for 16 hr., collected by centrifugation and washed in 0.9% NaCl. Reaction was carried out in double side-arm test tubes (Hughes & Williamson, 1952). In each was 10.0 ml. 0.2M-phosphate buffer, pH 6.5; 0.002M-nicotinamide; and 5 mg. dry wt. cells. The tubes were incubated at 40° and the reaction stopped by the addition of 1 ml. 2N-H₂SO₄. Ammonia was estimated after distillation and nesslerization. Nicotinic acid was estimated by microbiological assay after destruction of the remaining nicotinamide.)

Time after mixing (min.)	NH ₃ formed (μ moles)	Nicotinic acid formed (μ moles)
0	0.91	1.2
15	2.1	2.3
45	3.8	4.3
90	7.6	8.2
180	13.2	13.8

The maximum rate of deamidation of nicotinamide (1–2 μ moles/mg. dry wt./hr.) was some 200 times faster than the maximum rate of cozymase synthesis and appeared to be independent of the time for which the cells had been grown (up to 4 days). Cells grown on the semi-synthetic medium with adequate nicotinic acid and cells grown on the 'pronutrin' medium also deamidated nicotinamide at the same rate as the deficient cells. The rate of deamidation is therefore independent of the

cozymase content of the cells. In cells grown for 40–50 hr. the initial rate of cozymase synthesis from nicotinamide was 1.5–4.0 $\mu\text{m-moles/mg. dry wt./hr.}$ Under these conditions all added nicotinamide (100 $\mu\text{m-moles}$) was converted to nicotinic acid in the first 5–8 min. of the reaction. This means that the bulk of the cozymase synthesized by the cells after 2 hr. (8–10 $\mu\text{m-moles/mg. dry wt.}$) could not have been directly derived from the added nicotinamide.

The effect of constituents of the growth medium on cozymase synthesis by washed suspensions

To the washed suspensions, the constituents of the growth medium were added as shown in Table 8 under the conditions described for measuring

Table 8. *The effect of the constituents of the growth medium on the stimulation of glycolysis by nicotinic acid in washed suspensions*

(Glycolysis was followed manometrically. In the main compartment of each Warburg vessel was 2.0 ml. containing 0.02M-glucose, 0.02M- NaHCO_3 , 2.3 mg. dry wt. cells, and the substance tested in the final concentration shown below. Nicotinic acid ($5 \times 10^{-5}\text{M}$ final) was added from the side arm.)

Substance added	CO_2 ($\mu\text{moles/mg. dry wt./hr.}$)	
	Without nicotinic acid	With nicotinic acid
None	4.0	12.0
Acid casein hydrolysate (1.0%)	3.9	10.7
Tryptophan 0.04%, cystine 0.04%	4.0	12.0
Vitamin mixture (Barton- Wright, 1946)	4.0	12.0
Adenine, uracil, xanthine (0.02 mg. of each)	4.0	12.0
Guanine (0.02 mg.)	4.8	12.8
Glutamine (0.01 M)	3.8	11.4
Ribose (0.1 M)	3.8	11.4
NH_4Cl 0.01 M and salts B (Barton-Wright, 1946) 0.01 ml.	4.3	12.9

glycolysis. With the possible exception of guanine none of the constituents of the growth medium had any effect on the nicotinic stimulation of glycolysis. In some experiments guanine markedly decreased the length of the lag period. This effect could not be repeated at will in all batches of otherwise identical deficient cells and the effect was not investigated further. The rate of cozymase synthesis was not affected by addition of the vitamin mixture, tryptophan, glutamine, guanine, ribose, ammonium chloride, and casein hydrolysate. The omission of glucose completely stopped the synthesis of cozymase from nicotinic acid, nicotinamide and nicotinamide nucleotide. Neither pyruvic acid nor adenosinetriphosphate replaced glucose, but no other sources of energy were tested. In the presence of glucose the rate of cozymase synthesis from

nicotinic acid derivatives was the same when the reaction was carried out in air instead of in nitrogen, and when the bicarbonate was replaced by 0.05M-phosphate buffer, pH 7.8. Estimation of the glucose and lactic acid showed that the rates of glycolysis in air and in nitrogen with phosphate and bicarbonate media were identical. The rate of cozymase synthesis was not significantly altered by changes in the pH of the phosphate buffer from pH 5.8 to 8.2.

DISCUSSION

Catalytic effect of CoI. The catalytic effect of cozymase, expressed as a turnover number, i.e. mol. CoI/mol. acid formed/hr. (McIlwain, 1949) was 0.5 to 3.0×10^4 in washed suspensions of deficient cells. In cells supplemented with nicotinic acid, although the rate of glycolysis increased from two- to four-fold, the catalytic effect of cozymase remained the same. The value found for cozymase in washed suspensions agrees with the turnover number of nicotinic acid during growth under the conditions of the microbiological assay (McIlwain *et al.* 1949). This agreement indicates that deficiency in nicotinic acid does not greatly affect processes such as the synthesis of enzymes concerned in the glycolytic system as was found to be the case with biotin (Blanchard, Korkes, del Campillo & Ochoa, 1950). The catalytic effect of cozymase in cells grown upon medium containing an excess nicotinic acid, or in washed cells which had made cozymase from nicotinic acid was 3.0×10^3 , i.e. about ten times lower than in the deficient cells. The cells are therefore able to synthesize and bind about ten times more cozymase than is needed for glycolysis. The saturation level of cozymase (10 – $12 \mu\text{m-moles/mg. dry wt.}$) appeared not to be limited by supplies of the other parts of the cozymase molecule, ribose, adenine or phosphate, nor by constituents of the growth medium other than glucose.

Route of cozymase synthesis. In most species of bacteria, nicotinamide is as readily or more readily available for growth as is nicotinic acid (Knight, 1945). These findings are generally believed to indicate that the acid is amidated as the first step in the synthesis of cozymase. This idea is also supported by the studies on the inhibitory effects of pyridinesulphonic acid and pyridinesulphonamide (McIlwain, 1940) and by the present studies on the stimulation of glycolysis by nicotinamide and nicotinic acid in which the amide appeared to be more readily available than the acid. Further studies on the metabolism of nicotinamide showed, however, that even in young cells, where the synthesis of cozymase was most rapid, nicotinamide was deamidated some 50 to 100 times faster than cozymase was synthesized. In fact, in the older cells very little cozymase could have been synthesized from the

added nicotinamide and was made instead from the nicotinic acid formed by deamidation of the amide. But this does not rule out the possibility that nicotinamide is an intermediate in the synthesis of cozymase since reactions causing the breakdown of essential metabolites are of common occurrence in bacteria. For instance the breakdown of glutamine has been shown to occur in *Clostridium welchii* (Krebs, 1948; Hughes & Williamson, 1952) at a much faster rate and by a different reaction than it is synthesized (Fry, 1949). In erythrocytes, while nicotinic acid readily gives rise to cozymase, no cozymase was formed from nicotinamide (Handler & Kohn, 1943; Hoagland & Ward, 1942). In certain strains of *Pasteurella* and *Leuconostoc* nicotinic acid but not nicotinamide supports growth (Johnson, 1945; Koser & Kasai, 1950). These results could be taken as indicating that nicotinamide is not an intermediate in cozymase synthesis. The evidence as to whether nicotinic acid is amidated to form nicotinamide or whether nicotinic acid riboside is formed first and is then amidated must therefore remain uncertain. *Lb. arabinosus* does not seem to be suitable material on which to study this question, since no intermediates in the synthesis of cozymase from nicotinic acid accumulate during synthesis and excess nicotinamide is decomposed by the organism.

SUMMARY

1. Glycolysing washed suspensions of cozymase-deficient cells of *Lactobacillus arabinosus* 17-5 synthesized cozymase from nicotinic acid, nicotinamide riboside and nicotinamide ribotide.

2. The newly formed cozymase stimulated the rate of glycolysis by the cells from two- to five-fold. Maximum stimulation occurred in the presence of 1 to 5.0×10^{-6} M-nicotinic acid.

3. As judged by their effects on glycolysis, nicotinamide, nicotinamide riboside and ribotide were in older cells more readily available for cozymase synthesis than was nicotinic acid.

4. The rate of cozymase synthesis in any batch of cells was dependent on the added nicotinic acid concentration and was maximal at and above 5×10^{-6} M-nicotinic acid.

5. The maximum rate of cozymase synthesis (18 – 24 μ m-moles/mg. dry wt./hr.) occurred in cells grown for 18 – 24 hr. and fell progressively as the cells were grown for longer periods.

6. Cozymase synthesis and the uptake of nicotinic acid ceased when the cells contained from 10 to 12 μ m-moles cozymase/mg. dry wt.

7. Cozymase uptake and synthesis and the uptake of nicotinic acid and nicotinamide did not occur in the absence of glucose. No other constituent of the growth medium had marked effect on these processes.

8. Nicotinamide was deamidated rapidly (1 – 2 μ m-moles/mg. dry wt./hr.). This reaction was independent of the presence of glucose and of the cozymase content of the cells.

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Studies in Detoxication

44. THE METABOLISM OF BENZENE. THE MUCONIC ACID EXCRETED BY RABBITS RECEIVING BENZENE. DETERMINATION OF THE ISOMERIC MUCONIC ACIDS

BY D. V. PARKE AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 1

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Muconic acid (hexa-2:4-diene-1:6-dioic acid) was first discovered to be a metabolite of benzene by Jaffé (1909), who isolated it from the urine of dogs and rabbits receiving benzene. There are three geometrical isomers of muconic acid, namely, *cis-cis*, *cis-trans* and *trans-trans*, and in every instance in which the isolation of muconic acid from the urine of men and animals dosed with benzene has been reported, the isomer isolated has been the *trans-trans* form (Jaffé, 1909; Fuchs & Soós, 1916-17; Mori, 1918; Neumaerker, 1923; Thierfelder & Klenk, 1924; Drummond & Finar, 1938; Bernhard & Gressly, 1941). If muconic acid is formed *in vivo* by the opening of the benzene ring, then it would be expected on stereochemical grounds that the isomer produced would be the *cis-cis*-isomer; the oxidation of phenol or of catechol with peracetic acid does, in fact, give rise to *cis-cis*-muconic acid (Böeseken & Engelberts, 1931; Böeseken & Kerkhoven, 1932; Elvidge *et al.* 1950a).

The isolation of the *trans-trans*-acid from benzene urine, however, raised doubt as to whether benzene was the source of the acid, until Bernhard & Gressly (1941) showed that benzene containing deuterium gave rise *in vivo* to muconic acid containing deuterium. The *trans-trans*-acid could, however, arise from the *cis-cis*-acid by isomerization during the isolation procedures, but Drummond & Finar (1938) showed that this was probably not the case and they suggested that the isomerization probably took place *in vivo*.

That *cis-cis*-muconic acid is probably the first muconic acid to be derived biologically from benzene is suggested by the observation of Hayaishi & Hashimoto (1950) that an enzyme called pyrocatecase separated from a strain of *Pseudomonas* sp. oxidizes catechol to what they suggest is *cis-cis*-muconic acid, which was isolated in high yield in the crystalline state. Catechol is known to be a metabolite of benzene in the rabbit (Porteous & Williams, 1949a, b).

Recently, however, Elvidge, Linstead, Sims & Orkin (1950b) identified, for the first time, the third isomer of muconic acid, namely, the *cis-trans*-isomer, and they showed that recrystallization of the *cis-cis*-acid from water (but not ethanol) was sufficient to

convert it into the *cis-trans*-acid. This observation thus casts doubt on much of the earlier work which involved the use of the so-called *cis-cis*-acid.

The objects of the present work were to find methods of estimating and distinguishing between the three isomers of muconic acid, and to find out whether or not the urinary muconic acid was a mixture of isomers, since earlier workers could have lost the more soluble *cis*-isomers during purification. We shall show, however, that all the muconic acid of benzene urine from rabbits is almost certain to be the *trans-trans*-isomer.

EXPERIMENTAL

Preparation of materials

Melting points are uncorrected. *cis-cis*-Muconic acid was readily prepared by the oxidation of phenol by concentrated peracetic acid. (Elvidge *et al.* (1950a) used dilute peracetic acid, but in our hands better results were obtained with the concentrated acid.) The peracetic acid was prepared by cautiously mixing in the cold 10 ml. of 70% (w/w) H_2O_2 with 19 ml. of acetic anhydride, the molecular ratio of H_2O_2 to Ac_2O being 2:1.5, and keeping in cold water for 18 hr. Phenol (3 g.) was then added with shaking until it dissolved, and the mixture was kept in the dark. After 24 hr. the crystalline deposit of the *cis-cis*-acid was collected on a sintered-glass filter. Further quantities were obtained and filtered off each day by keeping the solution for 3 more days. The yield was 1.1 g. or 25%. The combined precipitates were washed with a little ethanol and twice recrystallized from ethanol. It formed colourless rhombs and had m.p. 194° as determined by the method of Elvidge *et al.* (1950b); the benzhydryl ester (Elvidge *et al.* 1950b) had m.p. 156° .

cis-trans-Muconic acid was prepared by twice recrystallizing the *cis-cis*-acid from hot water according to Elvidge *et al.* (1950b). It formed colourless prismatic needles from water, m.p. 190° , and its benzhydryl ester had m.p. 142° . A mixture of the *cis-cis*- and *cis-trans*-acids melted at $179-182^\circ$.

trans-trans-Muconic acid formed colourless minute prisms, m.p. 300° (decomp.) and was prepared according to Ingold (1921). The benzhydryl ester had m.p. 190° (cf. Elvidge *et al.* 1950b).

The lactones related to muconic acid were prepared by the methods of Elvidge *et al.* (1950b). The dilactone, butanolido-butanolide (3:7-diketo-2:6-dioxabicyclo[3:3:0]octane) formed pale brown prisms, m.p. 134° (Elvidge *et al.* (1950b) give m.p. 131°), depressed by admixture with the mono-

lactone to 90–95°. The monolactone, γ -carboxymethyl- Δ^{α} -butenolide, formed very pale brown rhombs, m.p. 110° depressed on admixture with the dilactone to 90–95° (Elvidge *et al.* (1950a) give m.p. 110.5–111.5°).

The colour reaction between muconic acid and phenol

When *cis-cis*- and *cis-trans*-muconic acids were heated at 130° for 5 min. with various phenols, together with a drop of conc. H_2SO_4 , coloured products were obtained which were soluble in water and organic solvents and fluoresced in ultraviolet light. The *cis-trans*-acid gave more intense colours than the *cis-cis*-acid, whereas the *trans-trans*-acid under the same conditions gave no coloured product at all. These observations are summarized in Table 1.

Table 1. Colours obtained by condensing *cis-cis*- and *cis-trans*-muconic acids with phenols at 130° for 5 min.

Condensing phenol	Colour of product	
	In acid solution	In alkaline solution
Phenol	Crimson*	Blue-green*
Resorcinol	Red-brown*	Red†
Catechol	Brownish violet	Olive green*
Quinol	Brown	Yellow brown‡
1-Naphthol	Brown*	Brown*
2-Naphthol	Brown*	Yellow†

* Light blue fluorescence in ultraviolet light.

† Green fluorescence in visible light.

‡ Blue fluorescence in visible light.

Identification of the muconic acids by the above colour reaction

The phenol most suitable for our purposes was phenol itself. The *cis-cis*- and *cis-trans*-acids give an intense crimson product in 5 min., whereas with the *trans-trans*-acid only a faint pink colour is obtained after several hours' heating. If 1 mg. of *cis-trans*-muconic acid is heated with 500 mg. phenol and 0.1 ml. conc. H_2SO_4 for 10 min. at 130° and the product dissolved in 50 ml. ethanol, this solution gives an extinction ($E_{1\text{ cm.}}$) of 0.7 on the Spekker absorptiometer using Ilford Spectrum green filter no. 604 and heat filter H 503. *trans-trans*-Muconic acid under the same conditions gives $E_{1\text{ cm.}} = 0.01$. With *cis-cis*-muconic acid the maximum colour is attained in 45 min. when $E_{1\text{ cm.}} = 0.76$, whereas with *trans-trans*-muconic acid in 45 min. $E_{1\text{ cm.}}$ is only 0.05.

If the temperature of the reaction is raised above 140°, *trans-trans*-muconic acid gives the same crimson colour as its isomers, maximum colour being attained in 20 min. at 160°. At 100°, the *cis-cis*- and *cis-trans*-acids give crimson colours after several hours' heating, but the *trans-trans*-acid gives no colour at all at this temperature. Thus by carrying out the test at different temperatures the *trans-trans*-acid can readily be distinguished from its isomers.

The two lactones, γ -carboxymethyl- Δ^{α} -butenolide and butanolido-butenolide, give the same colour at the same rate as *cis-trans*-muconic acid, and it is possible that one of these lactones is the precursor of the colour, although Elvidge *et al.* (1950a) were unable to convert the *trans-trans*-acid into these lactones. Elvidge *et al.*, however, point out that when the *trans-trans*-acid is heated at 160–165° with 95% H_2SO_4 , none can be recovered.

Quantitative estimation of muconic acid

The above colour reaction can be used for the quantitative estimation of muconic acid and the conditions for its use were investigated as follows.

Quantity of phenol and sulphuric acid. Experiments showed that the optimum amounts of phenol and conc. H_2SO_4 for development of the colour were 500 mg. and 0.1 ml., respectively, to about 1 mg. of muconic acid.

Effect of time of heating. In order to estimate the *cis-cis*- and *cis-trans*- in the presence of the *trans-trans*-acid it became necessary to carry out colour development at 100° for the former two acids and at 160° for the latter. The time of development of maximum colour at these temperatures

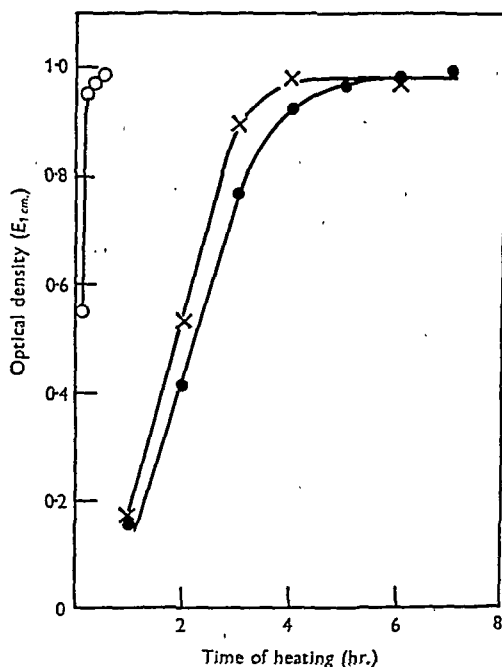


Fig. 1. The effect of time of heating on the production of the red colour obtained by condensing muconic acid (0.5 mg.) with phenol (0.5 g.) and concentrated H_2SO_4 (0.1 ml.) and then dissolving in 50 ml. absolute ethanol. x—x, *cis-trans*-Muconic acid condensed at 100°; ●—●, *cis-cis*-muconic acid condensed at 100°; ○—○, *trans-trans*-muconic acid condensed at 160°. E measured in the Spekker absorptiometer.

had to be determined. Muconic acid (0.5 mg.) was condensed with 0.5 g. phenol and 0.1 ml. conc. H_2SO_4 in corked tubes, at $100^\circ \pm 1^\circ$ for the *cis-cis*- and *cis-trans*-isomers and at $160^\circ \pm 2^\circ$ for the *trans-trans*-acid. Heating was continued for varying lengths of time, and then the coloured melts were dissolved in ethanol to a volume of 50 ml. The colour was measured in 1 cm. cells in the Spekker absorptiometer using colour filters as above. Maximum colour development was attained at 100° in 4 hr. with *cis-trans*- and the two lactones, and in 6 hr. with *cis-cis*-muconic acid. The *trans-trans*-acid gave no colour in 6 hr. at 100°, but gave the same maximum colour in 20 min. at 160° (see Fig. 1) as the *cis*-acids did in 6 hr. at 100°.

Effect of temperature. The above experiments showed that *cis-cis*- and *cis-trans*-muconic acids could be estimated by

heating with phenol and H_2SO_4 at 100° for 6 hr., whereas the *trans-trans*-acid could be estimated in the same way except that the heating had to be done at 160° for 20 min. In order to estimate the *cis*-acids in the presence of the *trans-trans*-acid, it was necessary to find out whether the *cis*-acids gave the same amount of colour at 160° as at 100° . It was found, however, that if the *cis*-acids were condensed at 160° , more colour was produced than at 100° . This was not seen with the *trans-trans*-acid, for temperatures above 160° merely accelerated the destruction of the colour produced by this acid. The effect of temperature on the development of colour with the three acids is shown in Fig. 2. In these experiments colour density was measured at $520 \text{ m}\mu$. (see below) in a Unicam spectrophotometer, the instrument finally adopted for all measurements of this colour. This extra colour

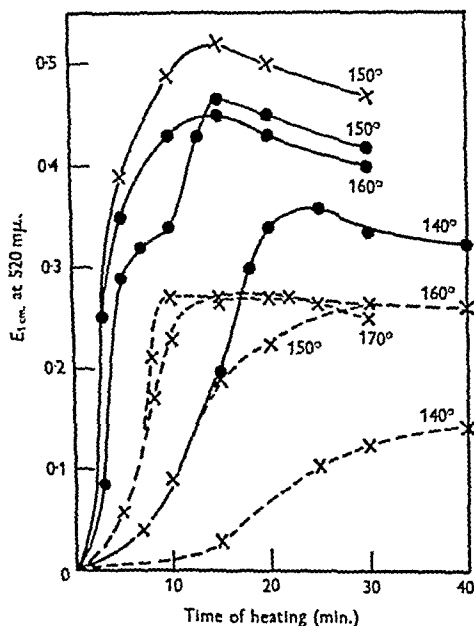


Fig. 2. The effect of temperature on the intensity of the red colour produced by heating the muconic acids with phenol and sulphuric acid. \times — \times , 0.1 mg. *trans-trans*-acid condensed for varying times at different temperatures and the product dissolved in 50 ml. ethanol; \bullet — \bullet , 0.1 mg. *cis-cis*-acid treated similarly; \times — \times , 0.1 mg. *cis-trans*-acid treated similarly. E measured in the Unicam spectrophotometer.

production at 160° with the *cis*-acids, although constant, showed that the *cis*-acids could not be readily estimated in the presence of the *trans-trans*-acid, although if the *trans-trans*-acid occurred alone it could be estimated. In benzene urine only one isomer, the *trans-trans*-, occurred, so that it could be readily estimated by this colour reaction. The elimination of this difficulty is described later (see below).

Stability of the colour. The colour in ethanol when exposed to air was stable for 15 min. after which time it slowly deteriorated probably due to absorption of moisture. E values at 0, 0.25, 0.5, 1, 2, 3 and 4 hr. were 0.78, 0.79, 0.75, 0.68, 0.55, 0.49 and 0.44 respectively. All readings were therefore carried out within 15 min. of development. Water (1–2%) reduced the intensity of the colour by a half.

Absorption spectrum of the colour and possible interference by other dicarboxylic acids. It seems possible from the mode of production of the colour, that a xanthone derivative is formed by the condensation of one of the lactones of muconic acid with two molecules of phenol. If this is true, then the possibility that other dicarboxylic acids can form such coloured complexes must be borne in mind especially when the method is applied to urine.

The absorption spectrum of the coloured complex in ethanol in the visible region showed a single maximum at $520 \text{ m}\mu$. with $E_{1 \text{ cm.}} = 0.56$ for a solution prepared from 0.2 mg. muconic acid (*cis-cis*- and *cis-trans*- by condensation at 100° and *trans-trans*- at 160°) and made up to 50 ml. with ethanol, measurements now being made with a Unicam spectrophotometer (see Fig. 3 curve A). All three isomers condensed as mentioned above gave the same $E_{1 \text{ cm.}}$

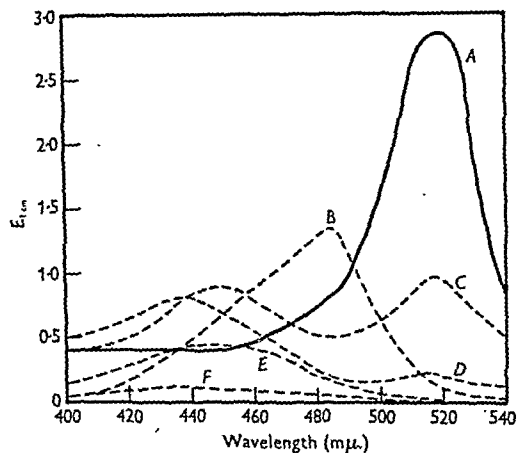


Fig. 3. Absorption spectra of the colours obtained by condensing at 160° for 20 min., certain organic acids with phenol and concentrated H_2SO_4 and dissolving the product in 50 ml. ethanol. Each curve corresponds to 1 mg. of the acid. A, *trans-trans*-muconic acid; B, oxalic acid; C, adipic acid; D, pimelic acid; E, succinic acid; F, suberic acid.

Common acids of the urine were first tested, namely benzoic, hippuric, phenylacetic, salicylic and *p*-hydroxybenzoic acids. When condensed with phenol and sulphuric acid at 160° , these acids give pale pink melts which dissolved with ethanol to give nearly colourless solutions. These acids did not interfere.

The dicarboxylic acids, oxalic, succinic, adipic, pimelic, suberic and azelaic acids were tested and all were found to give coloured melts at 160° , the spectra of which are recorded in Fig. 3. At $520 \text{ m}\mu$, the principal interference would come from adipic acid whose coloured complex (formed at 160°) at this wavelength has 33% of the absorption of the phenol condensation product of muconic acid. In the same way, pimelic and oxalic acids would interfere to the extent of 7 and 5% respectively. In the estimation of *cis-cis*- and *cis-trans*-muconic acids, that is with a condensation temperature of 100° , the interference would be much less being 1% for oxalic, 0.9% for adipic and 0.4% for pimelic acid.

The amounts of these dicarboxylic acids in rabbit urine are likely to be very small, although values are known only

for human urine. Hanson (1943) has reported traces of succinic, adipic and suberic acids in normal human urine, and gives the content of $\text{COOH}(\text{CH}_2)_n\text{COOH}$ as 15 mg./l. The succinic acid content of normal human urine has been reported by Weitzel (1947) as being 1.9–8.8 mg./l. in twelve subjects. According to Mori (1918) rabbits excrete about 4–9 mg./day of oxalic acid. The amounts of these dicarboxylic acids excreted, however, depend upon diet (Hanson, 1943; Weitzel, 1947).

Recovery of muconic acid from aqueous solutions. Solutions of *cis-cis*- or *trans-trans*-muconic acids (0.5 or 0.2 mg.) in 0.1N- Na_2CO_3 (1 ml.) were treated with 2N-HCl (0.05 ml.), and the solutions were evaporated at 105°. Phenol and H_2SO_4 were then added as above, and the tubes containing the *cis-cis*-acid heated for 6 hr. at 100° and those containing the *trans-trans*-acid at 160° for 20 min. The colour was measured as before. Recoveries were satisfactory (95–102%). The standard curves for the three acids are shown in Fig. 4.

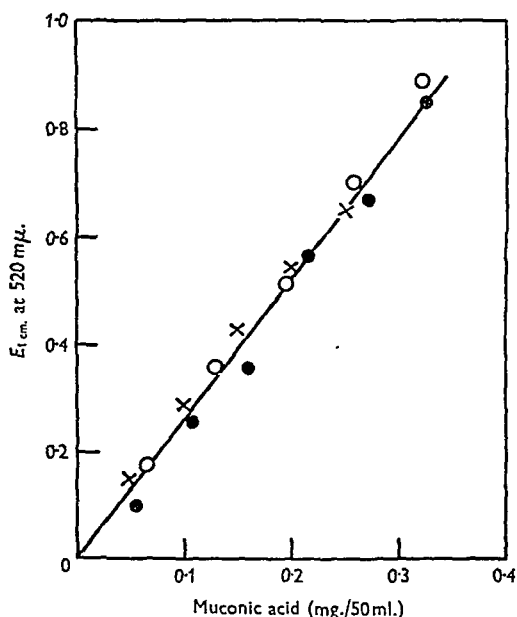


Fig. 4. Standard curve for the estimation of muconic acid, prepared by condensing muconic acid with phenol (0.5 g.) and conc. H_2SO_4 (0.1 ml.) at 100° for 6 hr. with the *cis-cis*- and *cis-trans*-acids and at 160° for 20 min. with the *trans-trans*-acid and then dissolving in 50 ml. absolute ethanol. $E_{1\text{ cm.}}$ at 520 $m\mu$. measured in the Unicam spectrophotometer. x, *cis-cis*; ●, *cis-trans*; ○, *trans-trans*.

Estimation of muconic acid in urine. The above method was now applied to rabbit urine. If this urine were dried at 105°, subsequent treatment with phenol and H_2SO_4 yielded dark-brown solutions. Much of this darkening could be avoided if the urines were dried at room temperature *in vacuo* in a desiccator over P_2O_5 . This also had the advantages of avoiding heat (which might cause isomerization), treatment with organic solvents and non-quantitative extraction procedures.

Normal urines from rabbits were collected in flasks containing 0.5 ml. 1% HgCl_2 . Each 24 hr. urine (slightly less than 100 ml.) was diluted with water to 100 ml. Solutions of

cis-cis- and *trans-trans*-muconic acids (5 mg./ml. in N- NaHCO_3) were added to 25 ml. of these urines which were then diluted with water so that the final concentration of muconic acid in each solution was 0.1 mg./ml. In this way, solutions of muconic acid corresponding to outputs of muconic acid of 20 and 100 mg./day were made up. 1 ml. portions of these diluted urine-muconic acid solutions in test tubes ($3 \times \frac{1}{2}$ in.) were then dried *in vacuo* over P_2O_5 at room temperature. Phenol (0.5 g.) and conc. H_2SO_4 (0.1 ml.) were then added to each dry residue. The tubes containing the *cis-cis*-acid were then heated at 100° for 6 hr. and those containing the *trans-trans*-acid at 160° for 20 min. The product from each tube was then dissolved in 50 ml. ethanol and filtered and the $E_{1\text{ cm.}}$ at 520 $m\mu$. then measured in the spectrophotometer. The recoveries were *cis-cis*- (at 20 mg./day), 78–85% and at 100 mg./day 95–98%; *trans-trans*- (at 20 mg./day), 89–95% and at 100 mg./day, 95–99%.

Recovery of muconic acid from benzene urine. Muconic acid (20 mg. in the least amount of N- NaHCO_3) was added to the 24 hr. filtered urine of a rabbit which had received a dose of 0.5 g./kg. of benzene, so that the concentration of added muconic acid was 0.2 mg./ml. Then 1 ml. samples of this urine were dried *in vacuo* and condensed with phenol as above. Blank estimations were carried out simultaneously on the benzene urine. The recoveries of added muconic acid were *cis-cis*-, 102–108; *cis-trans*-, 103–105; *trans-trans*-, 90–97%.

Muconic acid in the urine of rabbits receiving benzene

Benzene was administered orally to rabbits and the urine collected daily, made up to 100 ml. where necessary, and centrifuged. For each urine, eight tubes were set up containing 1 ml. of urine which was evaporated *in vacuo* in a desiccator over P_2O_5 . When dry, 500 mg. phenol and 0.1 ml. conc. H_2SO_4 were added to each tube. Three tubes were heated at 100° for 6 hr., three at 160° for 20 min. and two tubes (blanks) kept at room temperature for 6 hr. Each melt was then dissolved in ethanol, made up to 50 ml., filtered from any precipitated inorganic salts and the optical density measured at 520 $m\mu$. in the Unicam spectrophotometer. Normal urines collected before and after the feeding of benzene were treated in the same way. The colour (a general absorption) given by normal urine after heating at 100° for 6 hr. or at 160° for 20 min. remained very constant and gave a reading of $E_{1\text{ cm.}}$ approx. 0.1. In none of the experiments with benzene was there any increase in absorption at 520 $m\mu$. above that of the normal urines after condensation at 100° for 6 hr. From this it was concluded that the urine of rabbits fed benzene does not contain *cis-cis*- and *cis-trans*-muconic acids in amounts greater than 0.05% of the dose of benzene. The figure 0.05% is the limit imposed by the method. The amounts of *trans-trans*-muconic acid found are given in Table 2, which shows that at a dose level of 0.5 g./kg. 0.5% of the benzene appears as *trans-trans*-muconic acid. Fig. 5 shows an individual result.

Metabolism of muconic acid by micro-organisms

Since Evans & Smith (1951) have shown that *cis-cis*-muconic acid, but not the *cis-trans*- and *trans-trans*-isomers, is metabolized by certain micro-organisms, we had to consider the possibility that this acid might be formed and excreted by rabbits receiving benzene and then be rapidly

Table 2. *trans-trans-Muconic acid excretion by rabbits receiving benzene orally*

(*cis-cis*- and *cis-trans*-Muconic acids were absent from all these urines since no colour was obtained on prolonged heating at 100° with phenol and sulphuric acid. Dose level 0.5 g./kg. body wt. except for the two experiments at the foot of the table.)

Rabbit no.	Dose of benzene (g.)	Period of excretion (days)	<i>trans-trans</i> -Muconic acid excreted (mg.)	Benzene excreted as <i>trans-trans</i> -muconic acid (% of dose)
51	1.58	2	17.6	0.6
53	1.41	1	11.3	0.45
67	1.41	2	12.3	0.5
69	1.23	1	22.3	1.0
70	1.76	3	17.2	0.55
75	1.32*	2	4.9	0.15
82	1.32*	2	9.3	0.4
87	1.32*	1	14.7	0.65
69	2.46	3	17.1	0.4
70	3.52	3	22.0	0.35

* Urine collected in the presence of HgCl₂.

destroyed by micro-organisms infecting the urine. We found that the recovery of added *cis-cis*-muconic acid from very stale rabbit urine was negligible. In two experiments we recovered no *cis-cis*-muconic acid after 48 hr. when it was

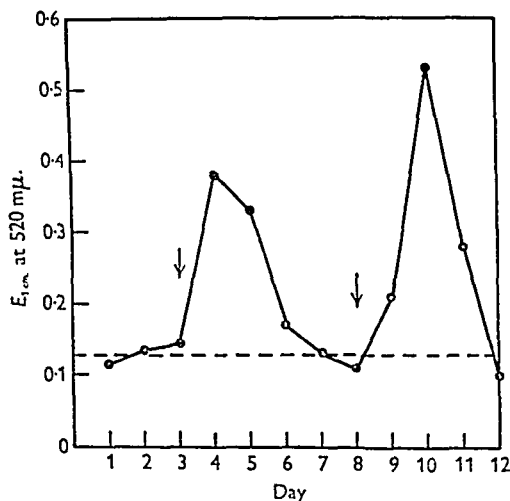


Fig. 5. The excretion of *trans-trans*-muconic acid by rabbit no. 70 which had received orally 1.76 g. of benzene on day 3 and 3.52 g. of benzene on day 8. The muconic acid excretion is expressed in terms of the E_{1cm} at 520 mμ. of the phenol-muconic acid colour in ethanol (see text).

added to stale urine in concentrations of 20 and 100 mg./100 ml., whereas the *trans-trans*-acid could be recovered almost quantitatively.

On culturing this urine on McConkey agar two predominant organisms were isolated. One was a late-lactose-fermenting paracolon bacillus and the other a faecal-type streptococcus (enterococcus). These organisms grew very scantily in 48 hr. on synthetic media of salts and vitamins which contained (NH₄)₂SO₄ and urea as sources of nitrogen and *cis-cis*-muconic acid as the source of carbon. None of the muconic acid was metabolized. These organisms however grew well on fresh, autoclaved rabbit urine containing *cis*-

cis-muconic acid, and in 96 hr. the faecal-type streptococcus metabolized 100% and the paracolon bacillus, 96% of the added *cis-cis*-muconic acid (0.5 mg./ml.). On subculturing 4 or 5 times on glucose nutrient agar media the organisms lost their ability to grow well in rabbit urine containing *cis-cis*-muconic acid.

It was concluded from these observations that in our experiments these organisms did not occur in sufficient numbers and did not metabolize *cis-cis*-muconic acid sufficiently rapidly to affect our results. In any case heavy growth of these organisms only occurred when rabbit urine had been allowed to stand for several days at room temperature. However, in order to eliminate this possibility, the urine from rabbits receiving benzene was collected in flasks containing 0.5 ml. of 1% HgCl₂. In this concentration HgCl₂ had no effect on the colour development. The results of these experiments (see Table 2) were essentially the same as those in which no HgCl₂ was used, and it was concluded that the absence of *cis-cis*-muconic acid was not due to its destruction by micro-organisms.

Isolation of muconic acid from benzene urine

A total of 7 g. of benzene (0.5 g./kg.) was fed by stomach tube with water to four rabbits. The 48 hr. urine (450 ml.) was made 0.2N with respect to HCl by adding conc. HCl. It was then saturated with (NH₄)₂SO₄ and continuously extracted for 6 hr. with peroxide-free ether. (In separate experiments it was shown that 150 mg. of *cis-cis*-muconic acid added to 100 ml. normal rabbit urine could be recovered almost quantitatively by ether extraction for 6 hr. at pH 2, as a muconic acid m.p. 185–186°, probably a mixture of *cis-cis* and *cis-trans*. No *trans-trans*-muconic acid was formed in this procedure.) The ethereal extract was decanted from a small quantity of wet tarry material and allowed to concentrate at room temperature. After cooling at 0° for several hours, the nearly colourless crystals (69 mg. or 0.6% of the dose) which had separated were filtered and washed with a little ether. Evaporation of the mother liquors yielded another 51 mg. of semi-crystalline material.

The colourless crystals were impure, for they began to melt at 180–185° and finally decomposed at 295–300°. On colorimetric assay, as described earlier, they contained no *cis-cis* or *cis-trans*-muconic acid and 20% of *trans-trans*-

muconic. This crude material was also tested microbiologically by Dr W. C. Evans of Aberystwyth (Evans & Smith, 1951) who reports that it contained no *cis-cis*-muconic acid.

Recrystallization of this material from ethanol eventually yielded pure *trans-trans*-muconic acid, m.p. 300° (decomp.); the yield of pure material in several experiments was 0.1–0.2% of the dose of benzene. The acid was further identified by the preparation of benzhydryl *trans-trans*-muconate, m.p. and mixed m.p. 190° (the *cis-cis*-ester melts at 156° and the *cis-trans*- at 142°). These isolation experiments strongly suggest that benzene urine contains only *trans-trans*-muconic acid.

Experiments on the injection of the three isomers of muconic acid

The estimation of cis-cis- (and/or cis-trans-) muconic acid in the presence of trans-trans-muconic acid. The colour reaction developed above for the estimation of the muconic acids could not be applied as such when more than one isomer occurred in the test solution, because of the different amounts of colour produced by the *cis*-acids at 100 and 160°. A solution of this difficulty, however, was found in the observation of Grundmann & Trischmann (1936) (cf. Elvidge *et al.* 1950b) that the *cis-cis*- and *cis-trans*-acids may be quantitatively converted to the *trans-trans*-acid by irradiation with ultraviolet light in the presence of small amounts of I_2 . The conditions of this transformation when the acids occurred in urine were therefore investigated. When these conditions were known the *cis*-acids could then be estimated in the presence of the *trans-trans*-acid by first estimating the *cis*-acids in a mixture by the colour reaction with phenol at 100°, then irradiating with ultraviolet light to convert the *cis*-acids into the *trans-trans* and finally estimating the total muconic acid as the *trans-trans*-isomer by the colour reaction with phenol carried out at 160°.

In pure solution it was found that 100% conversion of the *cis*-acids (5 ml. of 1 mg./ml. solution) to the *trans-trans*-acid occurred on irradiation from above, for 1–2 hr., of the solution containing 0.5 ml. of 0.1% ethanolic I_2 in small beakers placed 10 cm. below the ultraviolet lamp. In all experiments a Gallenkamp ultraviolet lamp was used, stated by the makers to contain a '125 watt mercury discharge unit enclosed by an ultraviolet glass filter from which there is practically no visible light, 95% of the radiation being at 3650 Å.' The solutions were thus in effect irradiated by light of wavelength 365 mμ, which is some distance from the absorption maximum of muconic acid which is at 260 mμ. However, irradiation of the solutions with a carbon arc lamp produced no better results than the Gallenkamp lamp.

It was also found that the isomerization could be carried out just as efficiently in $3 \times \frac{5}{8}$ in. test tubes kept upright and irradiated from above, as in open beakers. Furthermore, the isomerization took place equally well in dilute urine, dilute H_2SO_4 or $NaHCO_3$ and in the presence of $HgCl_2$ which was used as an antiseptic for the urines. The amount of I_2 used as a catalyst, however, was important. In the first place it was found that solutions of I_2 in KI interfered with the subsequent development of the red colour with phenol, but ethanolic solutions of I_2 were satisfactory because the I_2 could be removed by evaporation at the end of the irradiation period.

The effect of I_2 was investigated as follows. *cis-trans*-Muconic acid was dissolved in tenfold diluted normal rabbit urine to a concentration of 0.1 mg./ml. To 1 ml. of this

solution there was added from 0.05 to 0.2 ml. of 1% ethanolic I_2 solution. These solutions were irradiated with ultraviolet light for 2 hr. in $3 \times \frac{5}{8}$ in. test tubes, held upright 10 cm. below the lamp as mentioned above. The solutions were then dried *in vacuo* at room temperature over P_2O_5 and the residue condensed with 0.5 g. phenol and 0.1 ml. conc. H_2SO_4 at 160° for 20 min. as described for the estimation of *trans-trans*-muconic acid. The red colour was measured as before. Similar solutions without the irradiation were dried *in vacuo* and condensed with phenol at 100° as in the estimation of *cis-cis*-muconic acid. The results are shown graphically in Fig. 6, which shows that in 1 ml. of diluted rabbit

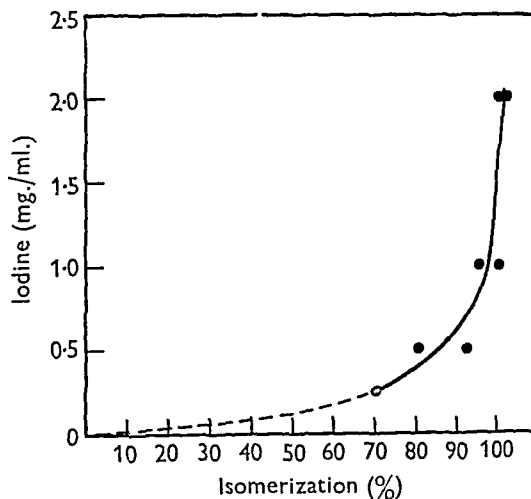


Fig. 6. The effect of the amount of iodine present on the isomerization by ultraviolet light of *cis-cis*- to *trans-trans*-muconic acid (0.1 mg./ml.).

urine 1 mg. of I_2 (0.1 ml. of 1% ethanolic I_2) gives 100% conversion of 0.1 mg. of the *cis*-acids to the *trans-trans*-acid on irradiation for 2 hr. This somewhat large amount of I_2 is necessary to allow for the consumption of I_2 by normal rabbit urine (approx. 4.5 ml. of 1% ethanolic I_2 /100 ml.) and for that lost by evaporation during the irradiation.

Mixtures of all three acids were now estimated in tenfold diluted rabbit urine. The procedure which was finally used for benzene urine was to take 1 ml. of urine appropriately diluted with water so as to contain 0.1 mg. of muconic acid and the procedure for the estimation of *cis-cis*-muconic acid was then followed. To another 1 ml. of the urine made up in the same way there was added 0.1 ml. of 1% ethanolic I_2 , then irradiated for 2 hr. with ultraviolet light and the procedures for the estimation of *cis-cis*- and *trans-trans*-muconic acids followed. With still another 1 ml. the *trans-trans*-estimation was carried out without irradiation. A blank estimation was also carried out on 1 ml. urine containing 0.1 ml. 1% I_2 but without muconic acid, condensations being carried out at 100 and 160°. With benzene urine the blank estimations were carried out on urine collected prior to dosing. Recovery experiments are given in Table 3.

Injection of trans-trans-muconic acid. The acid was dissolved in an equivalent amount of saturated aqueous $NaHCO_3$. Such a solution contained approx. 100 mg./ml. This solution was injected intraperitoneally into rabbits, each rabbit receiving 100 mg. muconic acid/kg. The urines were collected daily in flasks containing 0.5 ml. of 1%

Table 3. *Recovery of isomeric muconic acids from diluted rabbit urine*

(*E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol (see text). Corrected values (for 160° condensations) are obtained by subtracting the urine blank values.)

Material	Ultraviolet irradiation treatment	<i>E</i> at 520 m μ . Condensation temperature			Recovery (%)
		100° for 6 hr.	160° for 20 min.		
			Found	Corrected	
In aqueous solution (1 ml.):					
0.1 mg. <i>cis-cis</i>	None	0.275	0.53	—	—
0.1 mg. <i>cis-trans</i>	None	0.28	0.54	—	—
0.1 mg. <i>trans-trans</i>	None	0.02	0.27	0.27	—
In urine (1 ml.) + I ₂ (0.1 ml.):					
None (urine blank)	None	0.155	0.185	—	—
0.1 mg. <i>cis-cis</i>	2 hr.	0.185	0.445	0.26	95
0.1 mg. <i>cis-trans</i>	2 hr.	0.160	0.43	0.245	88
0.05 mg. each of <i>cis-cis</i> and <i>trans-trans</i>	2 hr.	0.135	0.485	0.300	109
0.05 mg. each of <i>cis-trans</i> and <i>trans-trans</i>	2 hr.	0.140	0.435	0.250	91

Table 4. *Excretion of muconic acid by rabbits after the intraperitoneal injection of the three isomeric muconic acids*

Muconic acid injected	Rabbit no.	Weight (kg.)	Dose (mg.)	Muconic acid recovered		
				As <i>cis-cis</i> or <i>cis-trans</i> (mg.)	As <i>trans-trans</i> (mg.)	Percentage of dose
<i>trans-trans</i>	75	2.8	250	0	137	56
	82	2.8	250	0	119	48
	87	2.2	175	0	86	49
	67	2.45	240	0	132	55
<i>cis-cis</i>	75	2.8	250	154	0	62
	82	2.8	250	179	0	72
	86	3.1	280	189	0	66
	51	2.4	240	145	0	61
<i>cis-trans</i>	75	2.8	250	150	0	60
	82	2.8	250	118	0	47
	86	3.1	280	159	0	58

Table 5. *Estimation of cis-cis-muconic acid in the urine of rabbits injected with the cis-cis-acid, and demonstrating the absence of the trans-trans-acid*

(Each rabbit was injected with 100 mg./kg. of *cis-cis*-muconic acid. *E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol.)

Rabbit no.	Ultraviolet irradiation treatment of urine	<i>E</i> at 520 m μ . condensation temperature	
		100° for 6 hr.	160° for 15 min.
75	None	0.415	—
	2 hr.	—	0.45
82	None	0.48	—
	2 hr.	—	0.48
86	None	0.51	—
	2 hr.	—	0.50

HgCl_2 . Each urine was then diluted to 1000 ml. with water, and 1 ml. of this diluted solution used for the estimation of muconic acid. Only traces of colour which were within the error of the method were obtained when the condensation

with phenol was carried out for 6 hr. at 100°, thus showing the absence of *cis-cis*- and *cis-trans*-muconic acid. The main colour production occurred on condensing with phenol at 160° for 20 min. In four experiments an average of 52% of the injected *trans-trans*-muconic acid was recovered in the urine as such within 24 hr. of dosing, there being no conversion into the other isomers (see Table 4). On continuous ether extraction of the urine after acidification, only the *trans-trans*-isomer was isolated, m.p. and mixed m.p. 300°; 80% of the original urine yielded 204 mg. of the acid which is equivalent to 36% of that injected.

Injection of cis-cis-muconic acid. In a similar manner, 100 mg./kg. of *cis-cis*-muconic acid was injected into rabbits and the urine collected and diluted as before. On developing the colour with phenol at 100° for 6 hr. an average of 66% of the injected acid was recovered unchanged (see Table 4). On developing the colour at 160° for 20 min. an increase in the amount of colour was observed, but after irradiation of the urine with ultraviolet light and then estimating *trans-trans*-muconic acid, the colour produced was the same as for the procedure for the *cis-cis*-acid (see Table 5). This indicated that urine contained no *trans-trans*-acid.

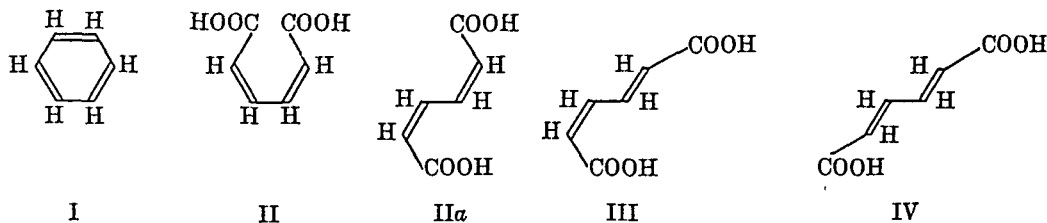
On continuous extraction of the urine with ether, the muconic acid isolated had m.p. 189° after two recrystalliza-

tions from ethanol. It was thus not *trans-trans*, but one or a mixture of the *cis*-acids.

Injection of cis-trans-muconic acid. In a similar way, *cis-trans*-muconic acid was injected and 55% was found again in the urine (Table 4). Ether extraction of the urine yielded the *cis-trans*-acid, m.p. 189–190°, after two recrystallizations from water (yield approx. 50% of dose).

DISCUSSION

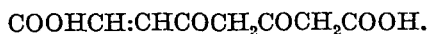
The discovery by Elvidge *et al.* (1950*b*) that mere recrystallization of *cis-cis*-muconic acid (II and II*a*) from water yields the *cis-trans*-isomer (III) makes much of the earlier biochemical work, in which the so-called *cis-cis*-muconic acid was used, difficult to interpret. If samples of *cis-cis*-muconic acid which had been crystallized from water were used, it appears very probable that earlier workers were handling either mixtures of the *cis-cis*- and *cis-trans*-isomers or the *cis-trans*-acid alone. This point is an important one since Evans & Smith (1951) have shown that the *cis-cis*-acid behaves biologically in a different manner from the *cis-trans*-acid.



(Elvidge *et al.* (1950*b*) suggest that the *cis-cis*- and *cis-trans*-acids probably occur in extended *s-trans*-configuration (II*a* and III). II is the *s-cis*-configuration of *cis-cis*-muconic acid showing its relationship to benzene, I.)

The present work confirms the findings of earlier workers that only *trans-trans*-muconic acid (IV) is found in the urine of rabbits receiving benzene (I). Furthermore, evidence has been presented to show that this isomer could not have arisen by isomerization in the urine of the *cis-cis*-isomer after excretion. It is also shown that if any *cis-cis*-muconic acid were excreted it could not have been destroyed, before estimations could be carried out, by micro-organisms infecting the urine. *trans-trans*-Muconic acid, therefore, appears to be a genuine metabolite of benzene and not an artifact. By our method of estimation the amount of benzene, at a dose level of 0.5 g./kg., excreted as this acid, is about 0.5% (range 0.15–1.0%) of the dose. It is thus a minor metabolite of benzene.

Recently Ravdin & Crandall (1951) have obtained a water-soluble enzyme system from rat liver which oxidatively cleaves homogentisic acid to 4-fumarylacetoacetate,



The configuration of the carbon-carbon double bond in this compound is *trans*, whereas the original configuration of this bond in the benzene ring of homogentisic acid is *cis*. Thus we have here another problem similar to that of the muconic acids.

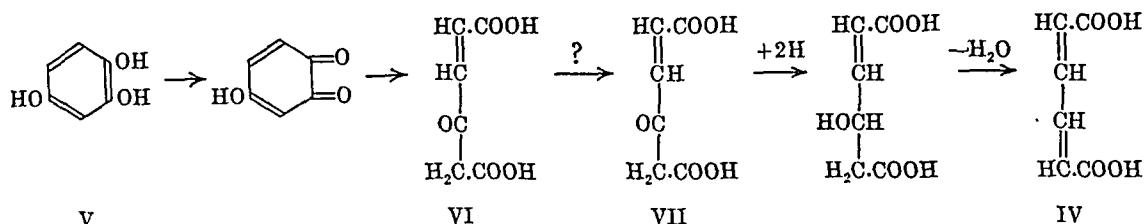
Drummond & Finar (1938) concluded from their experiments that *cis-cis*-muconic acid was not isomerized to the *trans-trans*-acid in the animal organism. On injection of '*cis-cis*-muconic acid' they recovered by isolation in two experiments 47 and 45% of the acid unchanged. With injected *trans-trans*-muconic acid they recovered by isolation an average of 56% of doses of 800 mg., and similar results were obtained by Mori (1918) who recovered 55–60%. In most of these experiments the yields were based on crude materials and are therefore probably high. Our experiments on the injection of the three isomers in rabbits confirm the views of Drummond & Finar. Whichever isomer is injected, the same isomer is excreted (when allowance is made for the easy isomerization in aqueous solution of the *cis-cis*- into the *cis-trans*-acid). We found that at a

dose level of 100 mg./kg., 50–65% of the acids were excreted unchanged in about 24 hr. The metabolites of muconic acid are not known, but it seems likely that the acid undergoes β -oxidation in the usual way, for Hensel & Reisser (1913) observed that in the perfused liver, muconic acid (presumably the *trans-trans*-acid as this was the only isomer known in 1913) gives rise to acetone. According to Raizon & Yamamoto (1940) perfusion of dog liver with the *trans-trans*-acid leads to the production of acetone bodies, but the *cis-cis*-acid does not. Mori (1918), however, reports that the *trans-trans*-acid does not increase the output of oxalic acid in rabbits.

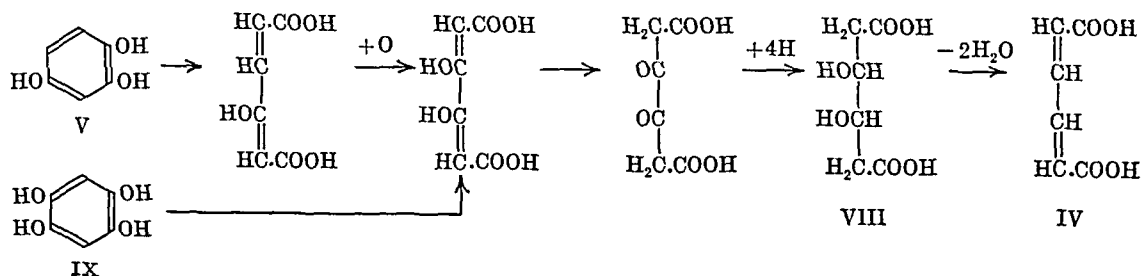
These results lead to the conclusion that *cis-cis*-muconic acid is not necessarily an intermediate in the metabolism of benzene in the animal body. Evans & Smith (1951), however, have suggested from simultaneous adaptation studies that *cis-cis*-muconic acid is an intermediate in the microbiological oxidation of phenol, catechol and benzoic acid to β -keto adipic acid. Hayaishi & Hashimoto (1950) have isolated *cis-cis*-muconic acid (or more probably *cis-trans*- in view of the treatment the material received during the isolation) from the oxidation of catechol by an enzyme from *Pseudomonas* sp. Thus *cis-cis*-muconic acid may be an intermediate in the bacterial oxidation of phenol,

although its microbiological production from benzene itself has not yet been studied.

Pathways of metabolism of benzene to *trans-trans*-muconic acid without the intermediate formation of the *cis-cis*-acid can readily be postulated. Thus hydroxyquinol (V) which is a metabolite of benzene (Porteous & Williams, 1949b) could give rise to fumarylacetic acid (VII) in the same way as homogentisic acid gives rise to fumarylacetoacetic acid (Ravdin & Crandall, 1951), although one would expect maleoylacetic acid (VI) as an intermediate. Reduction followed by dehydration of fumarylacetic acid could then give rise to *trans-trans*-muconic acid (IV) thus:



There are obvious difficulties in this postulate, such as the conversion of maleoylacetic to fumarylacetic acid. Another possibility is the formation of $\beta\beta'$ -dihydroxyadipic acid (VIII) which might give the *trans-trans*-acid on dehydration. Dihydroxyadipic acid could arise from hydroxyquinol or 1:2:4:5-tetrahydroxybenzene (IX) (this last phenol has not been found as a metabolite of benzene), thus:



According to Stanier, Sleeper, Tsuchida & Macdonald (1950) hydroxyquinol is probably not an intermediate in the bacterial oxidation of phenol and other compounds to β -ketoacid. The latter is a common intermediate in the bacterial oxidation of several aromatic compounds, but we have never detected it in the urine of rabbits receiving benzene.

It is possible that the solution of the stereochemical problems involved in the formation of *trans-trans*-muconic acid from benzene in the intact animal and of fumarylacetoacetic acid from homogentisic acid in tissue extracts may lie in the chemistry of unsaturated hydroxy compounds which undergo ready isomerization.

SUMMARY

1. A colorimetric method for the determination of the three geometrical isomers of muconic acid has been studied. The method is based on the production of a red colour by heating muconic acid with phenol and concentrated sulphuric acid and then dissolving the product in absolute ethanol.

2. The same amount of colour is produced on heating the *cis-cis*- and *cis-trans*-acids at 100° for 6 hr. as on heating the *trans-trans*-acid at 160° for 20 min. By this method the *cis*-acids can be distinguished from the *trans-trans*-acid and estimated

when they occur separately. This method has also been applied to urine.

3. When the *cis*-acids and the *trans-trans*-acid occur together the same colorimetric method can be used for their estimation, if the *cis*-acids are first estimated in the mixture and then converted to the *trans-trans*-acid by ultraviolet light followed by an estimation of the total muconic acid as the *trans-trans*-isomer.

4. By this colorimetric method it has been shown that rabbits receiving an oral dose of benzene excrete about 0.5% of the dose as *trans-trans*-muconic acid, there being none of the *cis*-isomers in the urine. *trans-trans*-Muconic acid was isolated from the urine and characterized as its benzhydryl ester.

5. It has been shown that if *cis-cis*-muconic acid had been excreted after administration of benzene it could not have been destroyed by bacteria.

6. The three isomers of muconic acid have also been injected into rabbits and no evidence was found for their *in vivo* isomerization, 50–65% of unchanged acid being recovered in the urine.

7. It is concluded that *trans-trans*-muconic acid is probably a true metabolite of benzene and is not produced *in vivo* from the *cis-cis*-acid. It is suggested that the *cis-cis*-acid need not be an intermediate in the formation of the *trans-trans*-acid in the animal body.

The expense of this work was in part defrayed by a grant from the Medical Research Council. We are grateful to Prof. R. Cruickshank and Dr R. Mendex for classifying the organisms found in stale rabbit urine, to Prof. W. C. Evans for the microbiological testing, of the urinary muconic acid and to Prof. R. P. Linstead, F.R.S., for access to information on the muconic acids prior to publication.

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The Separation of Esters of Choline by Filter-paper Chromatography

By V. P. WHITTAKER* AND S. WIJESUNDERA
Department of Biochemistry, University of Oxford

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The technique of paper chromatography (Consden, Gordon & Martin, 1944) has been applied in recent years to the separation of numerous classes of substances. It has now been applied to esters of choline in connexion with work on the identification of choline esters in tissue extracts and on the mechanism of enzymic reactions involving choline esters. Preliminary accounts have been given by Whittaker (1951) and Whittaker & Wijesundera (1951).

METHODS

Chromatographic procedure

Whatman no. 4 filter paper was used throughout. For preliminary screening of solvents, tall inverted beakers or wide-mouthed bottles with screw caps were employed as containers. Solutions of esters in water (10-50 µg. in 10 µl.) were delivered, 3 cm. from the lower edge, on to a rectangle of filter paper of suitable dimensions by means of an 'Agla'

micropipette (Burroughs Wellcome Ltd.). The paper was rolled into a cylinder whose axis was parallel to the intended direction of solvent flow, and the apposed ends of the paper secured by wire staples. The cylinders were then placed upright in a pool of solvent at the bottom of the container and irrigated by capillary ascent, a 10 cm. run taking 2-4 hr. Promising solvents were then tried with longer runs, using both upward and downward irrigation in containers of conventional design.

Reading of chromatograms

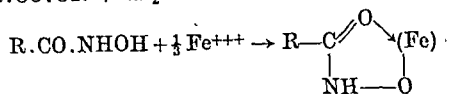
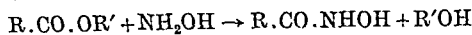
Esters were rendered visible on the paper by three methods, one specific for carboxylic esters and certain other compounds containing an acyl linkage (e.g. lactones, acyl phosphates), the other two relatively non-specific.

Carboxylic reagent

The first method employed a reaction first used by Feigl, Anger & Frehden (1934, cf. Feigl, 1939) as the basis of a spot test, and later applied to the quantitative estimation of acyl phosphates (Lipmann, 1946) and acetylcholine (Hestrin, 1949). The reaction involves the conversion of the carboxylic ester into a hydroxamic acid with alkaline hydroxylamine, followed by the formation of a purple

* Now at Department of Physiology, Cincinnati University College of Medicine, Cincinnati 19, Ohio, U.S.A.

complex between the hydroxamic acid and Fe^{+++} in acid solution:



In preliminary studies (by V.P.W.) the reagents of Hestrin (1949) were used. The papers were hung in a box inside a fume cupboard, and sprayed first with alkaline hydroxylamine, then, after 1 min., with acid FeCl_3 . An initial brown colour (due to $\text{Fe}(\text{OH})_3$) was replaced by yellow when sufficient acid had been delivered, the esters showing as purple spots. This method suffers from the following disadvantages: the spots are fugitive and must be outlined at once in pencil; the esters are soluble in the aqueous reagents and tend to diffuse away from their original positions; the reagents, being strongly alkaline and acid, are unpleasant to use. These disadvantages have been largely overcome, and the sensitivity considerably increased, by the use of non-aqueous reagents, which are also more stable.

Stock reagents. The following stock solutions are employed:

(A) Aqueous ethanolic hydroxylamine hydrochloride. Hydroxylamine hydrochloride (20 g.) is dissolved in water (50 ml.) and diluted to 200 ml. with 95% (v/v) ethanol. Stable when stored in the cold.

(B) Ethanolic KOH. The hydroxide (50 g.) is dissolved in the least quantity of water and diluted to 500 ml. with 95% (v/v) ethanol.

(C) Etheral acid FeCl_3 . Well powdered $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (10 g.) is dissolved in 20 ml. 10N-HCl and shaken with 300 ml. ether until a homogeneous solution results. Stable indefinitely in well stoppered bottles.

Spray reagents. These were made from the stock solutions as follows:

(I) Ethanolic alkaline hydroxylamine. Stock solution A is mixed with twice its volume of solution B and filtered from precipitated KCl. Stable for over 2 weeks in the cold.

(II) Stock solution C used directly.

Procedure. The dry chromatograms were clamped in a box, reagent I applied thinly and evenly with an atomizer supplied with compressed N_2 , dried briefly and sprayed with reagent II. The esters showed up almost at once as purplish spots on a yellow ground.

Sensitivity. As little as 0.5 μg . acetylcholine/cm.² gave a faint fugitive spot and 2 μg ./cm.² gave distinct spots which remained visible for several weeks. With weaker reagents, larger volumes have to be delivered on to the papers, thus making them wet and increasing the risk of diffusion. Etheral ethanolic hydroxylamine in place of reagent A gave fugitive spots.

Iodine method

Brante (1949) found that nitrogenous bases could be separated by paper chromatography and visualized as brown spots by immersing the paper in I_2 vapour. As pointed out by Marini-Bettolo-Marconi & Guarino (1950), this method is non-specific and can be applied to a wide variety of compounds. On exposing the treated paper to the air, the colour fades and the paper may then be treated with another reagent or assayed pharmacologically. We have found the iodine method particularly useful in conjunction with other methods, and exact coincidence was obtained in areas developed by the iodine and carboxylic methods.

Phosphotungstic acid-stannous chloride method

The method of Chargaff, Levine & Green (1948) for choline was found to be applicable to choline esters. It was found to be less convenient for our purposes than the other two methods, being less specific and less sensitive; moreover the washing-out stages were attended with a risk of damage to the chromatograms.

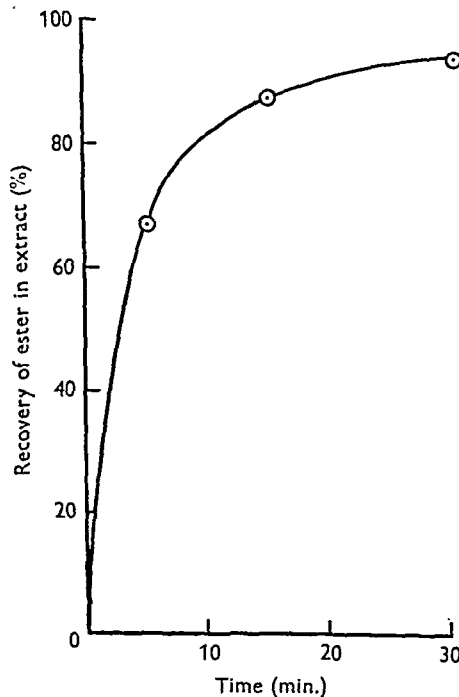


Fig. 1. Time required for extraction of acetylcholine from filter paper.

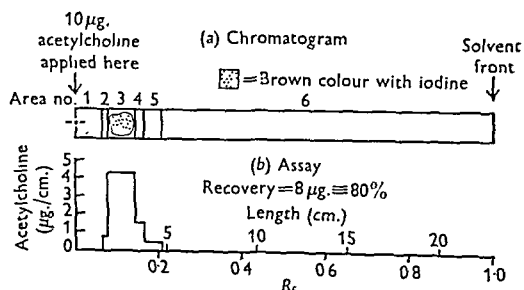


Fig. 2. (a) Chromatogram of acetylcholine. Solvent, *n*-butanol saturated with water (ascending). (b) Distribution of acetylcholine on chromatogram, as revealed by pharmacological assay (guinea pig ileum).

Pharmacological assay

Esters which are pharmacologically active can be localized by bioassay. Fig. 1 shows that elution of acetylcholine from filter paper with water is 93% complete in 30 min. In this experiment a series of spots, each containing 150 μg . acetylcholine chloride placed on filter paper, were run in *n*-butanol saturated with water. The papers were dried and the positions of the spots located by developing

control spots with iodine. Areas corresponding to the spots were then cut up and extracted in 3 ml. water each for various lengths of time. The acetylcholine content of samples withdrawn at 5, 15 and 30 min. was determined by the method of Hestrin (1949). Fig. 2 (a) is the tracing of a chromatogram of 10 μ g. acetylcholine run in *n*-butanol saturated with water. The chromatogram was cut into numbered areas; each area was cut up and eluted for 30 min. in 2 ml. of a saline solution acidified to pH 4, the pH at which acetylcholine has maximum stability. Each extract was assayed in the usual manner using a guinea pig ileum preparation. The result of the assay is shown in the lower half of the figure (Fig. 2b). It will be seen that good localization and recovery of the acetylcholine are obtained.

RESULTS

The R_F values of a number of esters in different solvents are presented in Table 1. Essentially the same values were obtained for an ester whether run alone or as a component in a mixture, but the values obtained in any one experiment were found to be influenced by the usual factors and rigid control of temperature, length of run and composition of solvent were necessary to secure uniform results.

Table 1. *Mean R_F values of choline esters and related compounds*

(Length of run: 25 cm. (upward); 35 cm. (downward); (a) denotes upward; (d) downward irrigation. Composition of solvents given as proportions by volume of constituents. Whatman no. 4 filter paper.)

Compound	<i>n</i> -Butanol saturated with water		<i>n</i> -Butanol- <i>n</i> - propanol-water	<i>n</i> -Propanol- water	<i>n</i> -Propanol- formic acid-water	<i>n</i> -Propanol-benzyl alcohol-water	
	(d)	(a)	(4:2:1)	(9:1)	(8:1:1)	(5:2:2)	
			(a)	(a)	(a)	(d)	(a)
Chlorides							
Acetylcholine	0.09	0.14	0.17	0.24	0.46	—	0.33
Acetyl- β -methylcholine	0.15	0.19	0.23	0.35	0.56	—	0.37
Propionylcholine	0.17	0.22	0.27	0.35	0.59	—	—
Butyrylcholine	0.22	0.28	0.29	0.43	0.66	—	0.46
Benzoylcholine	0.23	0.28	0.30	0.43	0.65	—	0.49
Lactylcholine	—	0.18	—	—	—	—	—
Valerylcholine	—	—	0.55	—	—	—	—
Choline	0.06	0.09	0.13	0.24	0.38	—	0.25
Perchlorates							
Acetylcholine	0.22	0.30	0.34	0.40	0.59	—	0.48
Acetyl- β -methylcholine	0.31	—	0.38	0.52	—	—	—
Propionylcholine	0.32	0.42	0.39	0.49	0.67	—	0.54
Butyryl- β -methylcholine	0.49	—	0.59	0.70	0.75	—	—
Lactylcholine	—	0.34	—	—	—	—	—
Succinylmonocholine	Unsatisfactory	—	0.04	Unsatisfactory	0.62	0.10	0.17
Succinyldicholine	Unsatisfactory	—	0.12	Unsatisfactory	0.30	0.19	0.30
Choline	0.21	0.24	0.31	0.39	0.60	0.50	0.46
β -Methylcholine	0.29	0.45	0.40	0.48	0.56	—	—

Increasing molecular complexity leads to increased R_F values. In *n*-butanol, for example, each additional CH_2 (starting with acetylcholine) increases the R_F by approximately 0.07; isomers, e.g. propionylcholine and acetyl- β -methylcholine ($[\text{Me}_3\text{N}^+\cdot\text{CH}_2\cdot\text{CHMe}\cdot\text{OAcjOH}^-]$), having closely similar values. The associated anion influences the R_F ; in *n*-butanol, the R_F 's of perchlorates are

0.13–0.16 above those of the chlorides. Increasing water content raised R_F values and reduced separation; with ethanol and acetone it also reduced the 'tailing' which occurred with the pure solvents. Pyridine-, collidine-, lutidine-, phenol-, benzyl alcohol-, furfuryl alcohol- or dioxan-water mixtures proved either unsatisfactory or inferior in resolving power to those included in the table. Solvents containing ammonia destroyed choline esters, which are markedly alkali-labile.

DISCUSSION

The results presented above show that it is possible to separate esters of choline using paper chromatography, the best solvents being the less freely water-soluble alcohols, either alone or in combination. Three methods of chemical identification have been described; one, using alkaline hydroxylamine and acid ferric chloride, is specific for compounds containing the acyl linkage and should be applicable to other esters of low volatility, and possibly also to acylphosphates and lactones, which can be estimated with the reagents (Kent, 1951).

SUMMARY

1. Choline esters can be separated from each other and from choline by the technique of paper chromatography. *n*-Propanol- and *n*-butanol-water mixtures, with or without the addition of a third component such as benzyl alcohol or formic acid, gave the best separations.

2. The esters were detected on the paper by an adaptation of the hydroxylamine-ferric chloride test for carboxylic esters, by the iodine method of Brante (1949), by the phosphotungstic acid-stannous chloride method of Chargaff *et al.* (1948)

and, in the case of acetylcholine, by pharmacological assay.

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The Metabolism of ^{14}C -Labelled Bicarbonate in the Cat

By H. L. KORNBERG, R. E. DAVIES AND D. R. WOOD

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, and Department of Pharmacology and Therapeutics, The University, Sheffield 10

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Studies on the metabolism of ^{14}C -labelled urea in mice (Leifer, Roth & Hempelmann, 1948; Skipper, Bennett *et al.* 1951) and cats (Kornberg, Davies & Wood, 1951b) have shown conclusively that urea is broken down in the mammalian body. Its rate of breakdown in these experiments has been measured from the rate of expiration of the ^{14}C -labelled carbon dioxide produced. In order to interpret the results of such studies on cats, it was necessary to have information on the rate of excretion from the blood of ^{14}C -labelled carbon dioxide, and on the rate of incorporation of ^{14}C into urea. The results of earlier studies on mice and rats injected with ^{14}C -labelled bicarbonate (Armstrong & Schubert, 1949; Armstrong & Zbarsky, 1949; Gould, Sinex, Rosenberg, Solomon & Hastings, 1949; Greenberg & Winnick, 1949; Schubert & Armstrong, 1949; Skipper, White & Bryan, 1949; Skipper, Nolan & Simpson, 1951; Skipper, Bennett *et al.* 1951) did not supply the required data, because of the great differences in body size between these animals and cats, and because the isotope in these experiments had been administered intraperitoneally.

Experiments are described in which ^{14}C -labelled sodium bicarbonate was injected intravenously into anaesthetized cats and the expired carbon dioxide continuously collected in a special respiration circuit. By this technique, information was obtained on the rate of expiration of labelled carbon dioxide from ^{14}C -labelled bicarbonate, and on the existence of exchange mechanisms between blood and tissue carbon dioxide in the animal. The results

of these studies are very similar to those obtained independently in human subjects (Hellman, 1951). From determinations of blood urea and bicarbonate it was also possible to measure the rate of incorporation of ^{14}C into urea synthesized during the experiment, and to confirm that the urea carbon is derived from carbon dioxide.

Part of this work has been communicated to the Biochemical Society (Kornberg, Davies & Wood, 1951a).

EXPERIMENTAL

Treatment of cats. A weighed cat was anaesthetized with ether followed by chloralose (75 mg./kg. body weight) and was placed on an operating table covered by a large stainless steel tray to avoid contamination of the laboratory. Cannulae were inserted into the trachea for collection of expired CO_2 , into the right external jugular vein for administration of intravenous injections, and into the right femoral artery for collection of blood samples during the course of the experiment. Both ureters were tied off. The animal was then connected through its tracheal cannula to the respiration circuit schematically represented in Fig. 1.

Respiration circuit. This system is so constructed that atmospheric pressure is maintained at the tracheal cannula, so that there is no resistance to free respiration. This is achieved by the two aquarium pumps P_1 blowing, and P_2 drawing air through the system at approximately 400 ml./min. Constancy of the pressure inside the circuit is controlled by three leaks L and a rubber balloon B which acts as a gas reservoir, these being placed at points which do not at any time come into contact with $^{14}\text{CO}_2$. Two soda-lime towers S remove atmospheric CO_2 from the incoming air, which can

be freely inspired by the cat through a valve inserted in the tracheal cannula. The expired CO_2 is absorbed in one or other of the two absorption trains A_1 or A_2 , only one of which is in circuit at any one time. They are changed by means of the two three-way taps T_1 and T_2 , and replaced by fresh absorption trains at frequent known intervals. The air, now again CO_2 -free, is passed down a water pump to avoid the possibility of accidentally contaminating the laboratory.

evolved was quantitatively absorbed in a slight excess of 0.3N-NaOH, in the apparatus described below. The liquid was adjusted to pH 9.3 by careful addition of 0.05N-HCl, using thymolphthalein as internal indicator. This gave a solution which was sufficiently alkaline to retain its $^{14}\text{CO}_2$ content, but which could be safely injected into the cat. Samples (1 ml.) of solutions prepared as above, containing 0.3–0.6 mg. carbon, and corresponding to 510 000, 700 000

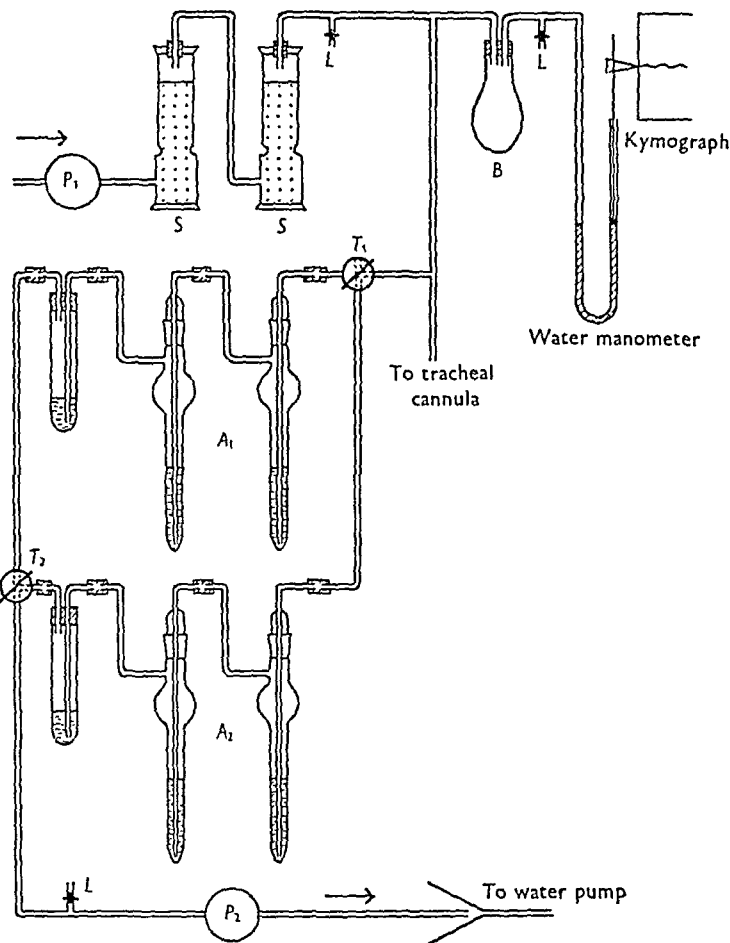


Fig. 1.

Fig. 1. Respiration circuit for absorption of expired $^{14}\text{CO}_2$. P_1 , P_2 , aquarium pumps; L , leaks with screw-clips; B , rubber balloon acting as gas reservoir; S , soda-lime towers; T_1 , T_2 , three-way taps; A_1 , A_2 , absorption trains (only A_2 is in circuit).

Fig. 2. A , vacuum transfer unit; B , cap for small tubes; C , small tube for $\text{Ba}(\text{OH})_2$; D , side-bulb cup; E , long pipette fitted with rubber bulb, for removal of liquid from absorption units.

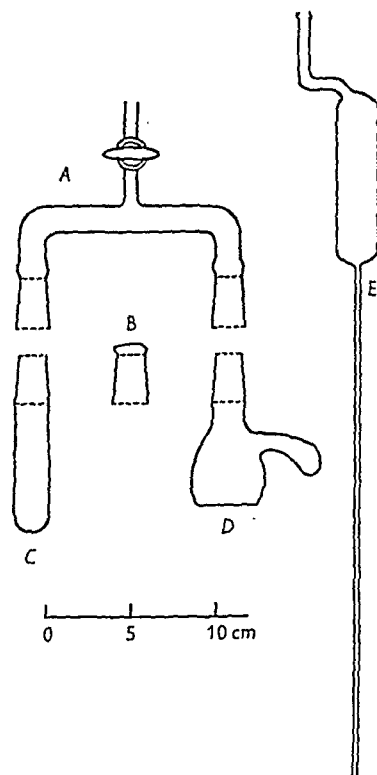


Fig. 2.

The absorption trains, A_1 and A_2 , consist of two all-glass units which are connected in series, and each of which contains 10 ml. approx. 6N-NaOH and 3–4 drops 0.75% (w/v) egg albumin. This latter substance acts as a frothing agent and, by forming a layer of alkaline bubbles in the units, ensures complete absorption of the CO_2 . The efficiency of absorption is checked by a third tube containing 5 ml. saturated $\text{Ba}(\text{OH})_2$, which solution should, and in fact does, remain unclouded throughout the period of absorption.

Preparation and injection of labelled materials. $\text{NaH}^{14}\text{CO}_3$ solutions were prepared from $\text{Ba}^{14}\text{CO}_3$. Samples (7–15 mg.) of $\text{Ba}^{14}\text{CO}_3$ (equivalent to 50–100 μc .) were acidified with lactic acid (British Pharmacopoeia) *in vacuo*, and the $^{14}\text{CO}_2$

and 1 020 000 counts/min. respectively under our conditions of radio-assay (see later), were injected quantitatively in the three experiments on HCO_3^- metabolism to be described.

Collection and analysis of samples

Carbon dioxide. When the ^{14}C -labelled material had been injected, the expired CO_2 was continuously collected over the next 4–5 hr., any one absorption train being used for periods varying from 5 to 20 min. The $\text{NaOH}\cdot\text{Na}_2\text{CO}_3$ solutions in the units were then quantitatively transferred by means of long pipettes with a rubber bulb at one end (Fig. 2E) to ampoules which were immediately sealed. The

units were then washed, fresh NaOH and frothing agent introduced, and the trains reassembled for use later in the experiment.

The liquid obtained from any one absorption train was made up to 50 ml. with water, and 1–2 ml. samples taken for analysis. All analyses were carried out in an apparatus modified by Davison from that of Barker (described by Kamen, 1948). These samples were pipetted into the main compartment of a side-bulb cup (Fig. 2D) and approximately 1 ml. lactic acid introduced into the side bulb by means of a bent Pasteur pipette. The side-bulb cup was attached to the transfer unit (Fig. 2A), a small tube (Fig. 2C) containing 3 ml. 0.22N-Ba(OH)₂ and 2 drops thymol blue, being attached to the other end. The system was evacuated, the tap closed and the contents of the side bulb tipped into the main compartment. CO₂ was rapidly evolved and rapidly absorbed in the Ba(OH)₂. Quantitative absorption was ensured by warming the side-bulb cup and transfer unit in the hand, the system being shaken continuously for 5 min. whilst the small tube was cooled under running cold water. CO₂-free air was admitted into the system, and the small tube, which contained BaCO₃, was detached and stoppered with a flat cap (Fig. 2B). The amount of CO₂ absorbed was determined by titration of the unneutralized Ba(OH)₂ with 0.20N-HCl. The precipitate was washed twice with water and once with acetone in a centrifuge, suspended in acetone, and plated on weighed aluminium disks, as described later.

At various times during the experiments, blood samples (1 ml.) were taken from the arterial cannula and received in stoppered tubes containing 0.2 ml. N-NaOH to prevent loss of CO₂. The tubes were stored at -12°. When required for analysis, the frozen clotted samples were thawed, homogenized with 1 ml. 1.7N-NaOH and 4 ml. water, washed into measuring tubes with 1 ml. water and the volumes read. About half the liquid was measured into the main compartment of a double-armed Warburg vessel, with 1 ml. 3M-acetate buffer, pH 5.0, in one side arm and 0.8 ml. jack bean urease solution buffered to pH 5.0 (Davies & Kornberg, 1951) in the other. The vessels were equilibrated at 25° and first the buffer was added from one side arm. The amount of gas evolved gave the 'total CO₂' content of the sample. The 'total CO₂' content of any tissue was defined as the acid-volatile CO₂, which included dissolved CO₂, H₂CO₃, HCO₃⁻, CO₃²⁻ and carbamino compounds. As alkali had been added initially to all samples, the 'total CO₂' at the time of analysis was present in the form of CO₃²⁻.

When equilibrium had again been reached, the jack bean urease was added from the other side arm. The urea in the sample was hydrolysed, and an equivalent amount of CO₂ evolved. These estimations were accurate to ±5%.

Another sample of the dilute blood homogenate, together with 1 ml. 0.125M-Na₂CO₃ (containing 1.5 mg. C, added as carrier) was added to a side-bulb cup, and 1 ml. 1.5N-H₂SO₄ was pipetted into the side bulb. The side-bulb cup was attached to one end of a transfer unit, a tube containing 3 ml. 0.22N-Ba(OH)₂ and 2 drops thymol blue attached to the other, the system evacuated and the cup contents mixed. The CO₂ evolved was assayed by the techniques described. The cup was detached, 2 ml. 0.125M-Na₂CO₃ and 0.5 ml. 3M-acetate buffer, pH 5.0, were added to the contents and the system was evacuated. Any trace of radioactive C remaining in the system as bicarbonate had thus been diluted with 3 mg. non-radioactive C, which was

then driven off on evacuation. CO₂-free air was admitted, 0.5 ml. 1.0% (w/v) urea solution was added to the contents of the cup, and 1 ml. jack bean urease solution pipetted into the side bulb. The cup was attached to one end of another transfer unit, a tube containing Ba(OH)₂ and thymol blue as above being attached to the other end. The system was evacuated and the cup contents mixed.

After 2 hr. all the urea in the sample had been decomposed, and the quantity and radioactivity of the ¹⁴CO₂ evolved was determined.

Tests showed that this procedure made it possible to determine independently highly active HCO₃⁻ and urea of low activity, present in the same sample of blood, without the results being vitiated by contamination.

Samples (1 g.) of cat tissues were taken during the experiments, blotted to remove adherent blood, and stored in weighed tubes containing 5 ml. 2N-NaOH to retain all acid-volatile CO₂. The tissues disintegrated on standing and were washed quantitatively into the main compartments of side-bulb cups with addition of 1 ml. 0.125M-Na₂CO₃ to act as a carrier. The tissue 'total CO₂' was decomposed by addition *in vacuo* of lactic acid from the side bulb, and the ¹⁴CO₂ evolved was measured.

Measurement of radioactivity

The washed BaCO₃ samples were suspended in acetone, shaken vigorously in the small stoppered tubes, and poured into stainless-steel cylinders which fitted firmly into the circular grooves of weighed aluminium plates. Acetone leaked slowly past the junction of cylinder and plate, leaving the fine precipitates of BaCO₃ (20–30 mg.) deposited on the plates as uniform layers of area 3.8 sq.cm. The cylinders were covered and allowed to dry at room temperature overnight. When dry, the cylinders were removed, the plates weighed, and the radioactivity assayed with a thin mica-window Geiger-Müller counter, corrections having been made for background counts and for the dead-time of the system. At least 5000 counts were taken for each sample, giving a standard error of less than 1.5%. The efficiency of counting was frequently checked by counting a uranium standard. There was no detectable loss for at least 3 days in the activities of the samples, kept covered in Petri dishes, through exchange of their ¹⁴C content with atmospheric CO₂.

All counts were corrected to infinite thickness for self-absorption in the BaCO₃ by means of an experimentally determined activity-saturation curve (cf. Calvin, Heidelberger, Reid, Jolbert & Yankwich, 1949), and the specific activities expressed as the corrected counts/min./mg. C. All analyses and counts were performed in duplicate and agreed to ±3%.

RESULTS

Expiration of ¹⁴CO₂

After intravenous injection of ¹⁴C-labelled sodium bicarbonate, the rate of expiration of ¹⁴CO₂ is initially very rapid, and corresponds to an exponential equation with three components. The curves obtained in our experiments were found empirically to correspond within 2% to the function

$$(100 - P) = 28.4e^{-0.243t} + 41.5e^{-0.0234t} + 30.2e^{-0.00119t},$$

where P is the percentage of injected ^{14}C left in the cat at t min. after the injection (Fig. 3). The three components therefore have half-lives of 2.85, 29.6 and 582 min. respectively. In the first 30 min., 50 % of the injected ^{14}C is expired, the total output over the 5 hr. experiments being 79–81 %.

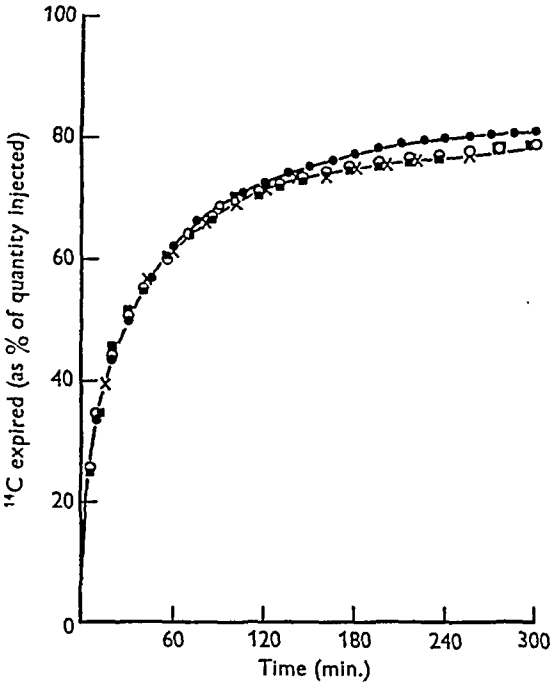


Fig. 3. Expiration of $^{14}\text{CO}_2$ after intravenous injection of $\text{NaH}^{14}\text{CO}_3$. \bigcirc — \bigcirc , cat A, 1.83 kg.; \bullet — \bullet , cat B, 1.55 kg.; \times — \times , cat C, 1.69 kg.; \blacksquare — \blacksquare , curve of $(100-P) = 28.4e^{-0.243t} + 41.5e^{-0.0234t} + 30.2e^{-0.00119t}$, where P is the percentage of injected ^{14}C remaining in cat after t min.

Semi-logarithmic plots of the specific activities of the expired $^{14}\text{CO}_2$ against time (Fig. 4) give smooth curves. Analysis of the radioactivity of the blood 'total CO_2 ' showed that the specific activities of expired CO_2 and blood 'total CO_2 ' are identical at any time, and these curves thus also represent the change in the specific activities of blood 'total CO_2 ' with time. The three curves obtained in our experiments cut the ordinate at specific activities of approx. 16 000, 16 000 and 28 000 counts/min./mg. carbon respectively. As the blood volume of the cat is approx. 70 ml./kg. body weight, and as the amounts of bicarbonate injected were negligible, this corresponds in each case to a blood 'total CO_2 ' concentration of 24–27 mm. This is the 'total CO_2 ' content of blood (Krebs, 1950) and shows that only at zero time is the radioactive material present entirely in the blood. After 3 hr. the rate of expiration of $^{14}\text{CO}_2$ is nearly constant and is less than 2 % of the injected isotope/hr. The blood and various soft tissues, which were weighed, have low, slowly decreasing, activities in their 'total CO_2 ' at this time

(Table 1), and after 4 hr. together contain approx. 1 % of the injected ^{14}C , i.e. approx. 5 % of the ^{14}C still remaining in the cat. The activity of bone is 15–40 times that of any other tissue, and, since bone

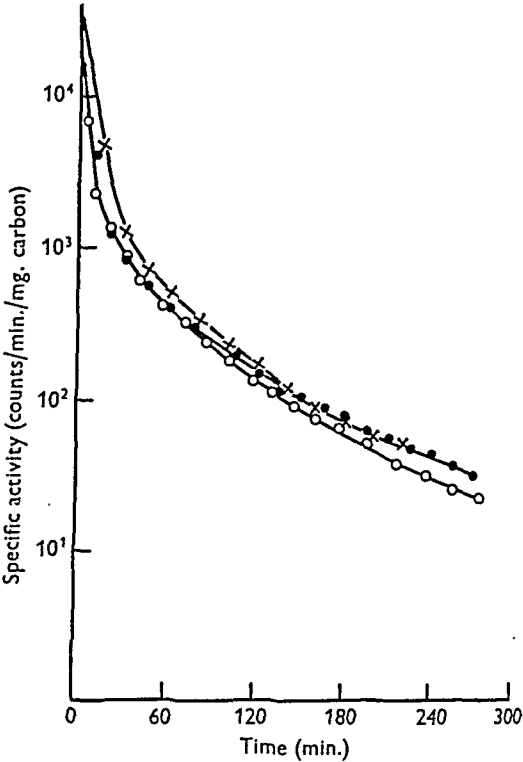


Fig. 4. Changes in specific activity of expired $^{14}\text{CO}_2$ after intravenous injection of $\text{NaH}^{14}\text{CO}_3$. \bigcirc — \bigcirc , cat A, 1.96 kg.; \bullet — \bullet , cat B, 1.55 kg.; \times — \times , cat C, 1.69 kg.

forms approx. 18 % of the body weight of the animal (Custor, 1873) over 6 % of the injected ^{14}C , i.e. over 30 % of the isotope still remaining in the cat, is present in this tissue at this time.

Table 1. Radioactivity of 'total CO_2 ' of tissues after intravenous injection of $\text{NaH}^{14}\text{CO}_3$ into a cat

(Cat, 1.5 kg., received 1×10^6 counts/min. intravenously at time 0.)

Time (min.)	Tissue radioactivity (counts/g. wet wt.)				
	Blood	Jejunum	Muscle	Marrow	Femur
32	360	143	—	—	—
58	155	84	85	—	—
91	82	43	—	—	—
120	51	28	23	—	—
150	30	15	—	—	—
182	21	11	10	—	—
240	13	6	5	11	220

Urea synthesis

As the ureters of the experimental animals were tied at the start of each experiment, no ^{14}C was lost in the urine, and urea synthesis could be followed

both by the rise in the concentration and radioactivity of blood urea (Table 2). The urea synthesized in the early stages of the experiments is formed from CO₂ which is in equilibrium with blood bicarbonate of high specific activity, and the major part of the incorporation of ¹⁴C into urea occurs in the first 40 min., the blood urea by this time containing 2.0 % of the ¹⁴C injected. After 5 hr. 3.1 % of the administered isotope was incorporated into urea.

Table 2. Concentration and specific activity of blood urea after intravenous injection of Na¹⁴HCO₃

(Cat I (1.55 kg.) received 5.1×10^5 counts/min., cat II (1.83 kg.) 7.0×10^5 counts/min., at time 0. Ureters tied.)

Cat no.	Time (min.)	Blood-urea concentration (mg./100 ml.)	Specific activity (counts/min./mg. carbon)	Percentage of injected ¹⁴ C incorporated in urea
I	0	31.0	0.00	0.00
	5	31.0	56.8	0.80
	16	32.3	102	1.50
	25	32.6	117	1.74
	40	35.5	124	2.00
	70	36.0	145	2.37
	100	36.5	154	2.56
	130	42.0	150	2.67
	160	40.0	154	2.80
	192	44.0	143	2.87
	220	47.5	135	2.91
II	250	47.7	136	2.96
	280	50.1	134	3.06
	0	62.5	0.00	0.00
	20	65.0	64.5	1.64
	35	70.0	70.1	1.92
	48	68.2	78.7	2.10
	78	69.7	88.7	2.42
	115	70.6	94.7	2.62
	153	77.5	91.4	2.77
	198	77.7	94.7	2.88
	253	80.0	94.5	2.96
	285	84.0	94.4	3.10

The total quantity of urea carbon synthesized during the experiments was obtained by multiplying the rise in plasma urea-carbon concentration the 'urea space' of the cat. The 'urea space', i.e. total volume of water in which the urea of the ly is dissolved, has been found to be 65 % of the

body weight of the cat (Kornberg *et al.* 1951b). Table 3 shows that the ratio

$$\frac{\text{carbon incorporated into urea}}{\text{carbon incorporated into urea} + \text{carbon expired as CO}_2}$$

was 0.037 or 0.038. Comparison of the distribution of radioactivity in urea and expired CO₂ show that the ratio

$$\frac{{}^{14}\text{C-urea synth. from labelled bicarbonate}}{{}^{14}\text{C-urea synth. from labelled bicarbonate} + {}^{14}\text{CO}_2 \text{ expired}}$$

was also 0.037 or 0.038. This means that within the experimental error of the average values ($\pm 2\%$) the urea carbon synthesized during the experiments must have been derived solely from carbon in equilibrium with blood bicarbonate, and confirms the results obtained *in vitro* by Grisolia & Cohen (1948), and *in vivo* by Mackenzie & du Vigneaud (1948), Sprinson (1949), and Armstrong & Zbarsky (1949).

DISCUSSION

Rate of expiration of ¹⁴CO₂

When ¹⁴C-labelled sodium bicarbonate or carbonate is injected intraperitoneally into mice or rats, ¹⁴C is rapidly expired as ¹⁴CO₂. Skipper *et al.* (1949), Skipper, Nolan *et al.* (1951), Skipper, Bennett *et al.* (1951) using mice, recovered 96 % of the injected dose in this form within 24 hr., 92.8 % having been expired within the first hour and over 50 % in the first 10 min. The total elimination in rats is very similar to that in mice, recoveries of 93–98 % being recorded (Armstrong & Zbarsky, 1949; Gould *et al.* 1949; Greenberg & Winnick, 1949) but the rate of expiration is slightly lower, 50 % of the injected ¹⁴C appearing as ¹⁴CO₂ in the first 18 min. (Gould *et al.* 1949). The experiments described in the present paper show that in cats only 50 % of the injected ¹⁴C is expired in the first 30 min., and that the total isotope expired in 5 hr. is 79–81 % of that injected. It is of interest that both the rate and total output of ¹⁴CO₂ was less in cats than in mice and rats, even though the material was injected intravenously into the cats, in contrast to the intraperitoneal injections

Table 3. The source of the carbon of urea

Cat no.	I	II
Body wt. (kg.)					1.55	1.83
Urea 'space' (ml.)					1010	1190
Blood-urea concentration (mg./100 ml.):						
At start						
At end					31.0	62.5
(A) Total urea synthesized (mg. carbon)					50.1	84.0
(B) Total respiratory CO ₂ output (mg. carbon)					44.0	59.0
(A)					1120	1500
(A) + (B)						
(A') ¹⁴ C fixed as urea (counts/min.)					0.037	0.038
(B') ¹⁴ CO ₂ expired (counts/min.)					15 600	21 700
(A')					411 000	547 000
(A') + (B')					0.037	0.038

with the smaller animals. The rate of excretion of $^{14}\text{CO}_2$ after intravenous injection of labelled sodium bicarbonate can be formulated as an exponential equation with three components; at no time does it correspond to the first-order reaction described by Gould *et al.* (1949) after intraperitoneal injection of labelled bicarbonate into rats.

The 'bicarbonate pool'

The data of Gould *et al.* (1949) show that the specific activity of the expired $^{14}\text{CO}_2$ in their experiments falls at a uniform rate for the first 60 min., and that the initial specific activity obtained by extrapolation of the curve to zero time indicates a 'bicarbonate pool' of 2m-moles/100 g. rat. As the total acid-volatile CO_2 in the extracellular and intracellular fluid of an animal of this size amounts to only 1m-mole, the authors stated that 'the C^{14}O_2 must have rapidly entered into mobile equilibrium with 1m-mole of CO_2 elsewhere in the body. One possibility is rapid exchange with the CO_2 of bone, and another is incorporation into dicarboxylic and tricarboxylic acids.' This conclusion and the earlier one of Brues & Buchanan (1948) that the total mass of carbon in mobile equilibrium with CO_2 in the body is greater than the total amount of CO_2 and bicarbonate in solution, is confirmed by our present findings, which provide evidence for the existence of exchange mechanisms between blood and tissue 'total CO_2 '. After injection of labelled bicarbonate, ^{14}C is taken up into the tissues at varying rates from blood bicarbonate of initially very high radioactivity. The ^{14}C content of any tissue reaches a maximum when the specific activities of blood and tissue 'total CO_2 ' become nearly equal.

In these early stages of the experiments the distribution of ^{14}C amongst the blood and tissues will thus be in the order of their rates of CO_2 exchange. The 'fixed' isotope is released again into the blood at rates governed by many factors, such as the 'total CO_2 ' content, diffusion constants, blood supply, and metabolic activity of the tissue. The concentration of 'total CO_2 ' in cat blood is approx. 25 mM and a 2 kg. cat thus contains about 45 mg. carbon as acid-volatile CO_2 in its blood. Since, however, the rate of expiration of CO_2 is of the order of 90 mg. carbon/15 min. an amount of carbon equivalent to the total amount in the blood is expired about every 7 min. This rapid removal through the lungs of ^{14}C from the blood will preferentially deplete of their isotope content those tissues with the highest rates of exchange of CO_2 , so that after 4 hr. the distribution in the tissues of the ^{14}C still remaining in the cat is, in general, in inverse order of these exchange rates (Table 1, cf. Skipper, Nolan *et al.* 1951). Of the ^{14}C injected as labelled bicarbonate in the present experiments, after 4 hr. only about 1% could be accounted for as the residual labelled bicarbonate of

the blood, muscles and viscera, but approx. 6% was 'fixed' in bone. This large amount of incorporation is mainly due to the high 'total CO_2 ' content of bone. These facts present striking evidence for the occurrence of CO_2 exchange mechanisms in the body.

Further evidence is provided by the data on the expiration of $^{14}\text{CO}_2$ from the cat. As already stated, the graph of the percentage of injected ^{14}C expired against time is an exponential curve with three components (Fig. 3). These have half-lives of 2.85, 29.6 and 582 min. respectively, and, though the detailed nature of the three components is uncertain, they show that the 'bicarbonate pool' is certainly not homogeneous. This conclusion is also indicated when attempts are made to calculate the 'bicarbonate pool' of the cat by the procedure of Gould *et al.* (1949). The semi-logarithmic plot of specific activity against time (Fig. 4) is a smooth curve in each experiment, which at no time becomes linear. Attempts to calculate the 'bicarbonate pool' by dividing the ^{14}C remaining in the cat at any point by the ^{14}C -bicarbonate concentration of the blood at that point give a steadily rising 'pool' which after some time exceeds the body weight of the animal. The underlying assumption of this procedure, that all the ^{14}C remaining in the cat at any time is rapidly miscible with, and has the same specific activity as, blood bicarbonate, must therefore be erroneous, and 'fixation' of ^{14}C in some form not rapidly miscible with blood bicarbonate must therefore have occurred. This is supported by the fact that in the long-term experiments with mice and rats the 'total CO_2 ' of bone and other tissues of low metabolic activity (cf. Skipper *et al.* 1949; Skipper, Nolan *et al.* 1951; Neuberger, Perrone & Slack, 1951) have specific activities much higher than that of blood.

The synthesis of urea

Recent studies on the breakdown of labelled urea in mice (Leifer *et al.* 1948; Skipper, Bennett *et al.* 1951) and cats (Davies & Kornberg, 1950; Kornberg *et al.* 1951b) have shown that urea is not a stable end product of nitrogen metabolism, but is broken down in the body: after intravenous injection of ^{14}C -labelled urea into cats, approx. 2% of the isotope was expired in 5 hr. as $^{14}\text{CO}_2$. This slow evolution of CO_2 from urea may be likened to the processes occurring in a 'pool' with a low rate of CO_2 exchange, and the mechanisms of incorporation of ^{14}C into urea from ^{14}C -labelled bicarbonate should thus be similar to those postulated above. After 5 hr., 3.1% of the ^{14}C -labelled bicarbonate is present as urea carbon, but though the rate of urea synthesis was uniform throughout the experiments (Table 2), 50% of this incorporation occurred in the first 30 min. Most of this 'fixation' of ^{14}C thus took place when the radioactivity of blood bicarbonate was very high, the rate of release of 'fixed' ^{14}C being

governed by the rate of breakdown of urea. These facts thus fully support the views expressed above on the 'fixation' of ¹⁴C in other tissues of the body.

It was first shown by Grisolia & Cohen (1948) *in vitro* and by Mackenzie & du Vigneaud (1948) *in vivo* that the specific activities of bicarbonate carbon and urea carbon are the same, and that the urea carbon is, within the experimental error, derived wholly from CO₂. The present findings (Table 3) confirm this view; this means that in our experiments with fasted cats less than 2% of the urea synthesized was formed from precursors other than labelled bicarbonate, such as arginine derived from the diet or from protein breakdown.

SUMMARY

1. Apparatus and techniques are described for *in vivo* studies of the metabolism of ¹⁴C-labelled bicarbonate in the cat.

2. The rate of expiration of ¹⁴CO₂ after intravenous injection of NaH¹⁴CO₃ is initially very rapid, 50% being expired in the first 30 min., the total expired in 5 hr. being 79–81% of the injected ¹⁴C. The rate of expiration can be represented by an

exponential equation with three components of half-lives 2.85, 29.6 and 582 min. respectively.

3. Of the ¹⁴C injected, 3.1% was incorporated into urea in 5 hr., 50% of this incorporation occurring in the first 30 min. Within the experimental error ($\pm 2\%$) all the urea carbon synthesized was derived from carbon in rapid equilibrium with blood bicarbonate.

4. After 4 hr. approximately 6% of the injected ¹⁴C was present in bone, the carbon of which exchanges relatively slowly with blood bicarbonate. The blood, muscles and viscera contained approximately 1% of the isotope at that time.

5. These results show that the bicarbonate pool is not homogeneous, but consists of several component CO₂-exchange mechanisms operating at widely differing rates.

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Studies on the Metabolism of Progesterone and Related Steroids *in vitro*

2. FACTORS INFLUENCING THE METABOLISM OF PREGNANE-3 α :20 α -DIOL BY RAT-LIVER HOMOGENATES, AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

By J. K. GRANT

Department of Biochemistry, University of Edinburgh

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In the first paper of this series (Grant & Marrian, 1950) experiments were described which showed that rat and rabbit liver preparations were able to metabolize pregnane-3 α :20 α -diol (pregnanediol) to unknown products *in vitro*. No metabolism occurred under anaerobic conditions unless a hydrogen acceptor (methylene blue) was added. The metabolism was uninfluenced by cyanide, and the evidence was compatible with the view that the agent in liver responsible for the metabolism of pregnanediol was a dehydrogenase. The possibility that more than one enzyme was involved was not excluded.

The work has now been extended with rat-liver homogenates, which have been employed in an investigation of factors influencing the metabolism of pregnanediol. The ultimate aim of these experiments was to obtain a system of optimum activity which could be used in an attempt to isolate products of metabolism in a large scale experiment.

It has been shown that the metabolism of pregnanediol by liver homogenates is increased by coenzyme I. In attempts to obtain the product or products of metabolism in large scale experiments pregnan-3 α -ol-20-one was isolated, but in amounts only sufficient to account for approximately 10% of the metabolized pregnanediol.

EXPERIMENTAL

Materials and methods

Riboflavin and aneurin pyrophosphate were obtained from Roche Products Ltd.; adenosinetriphosphoric acid (ATP) was a gift from Dr H. Bergel, glutathione from Dr A. P. Meiklejohn and adenosinemonophosphoric acid (AMP) from Dr I. D. E. Storey. Coenzyme I (CoI) was prepared in the laboratory by the method of Le Page (1949), and its purity as determined spectrophotometrically (Le Page, 1947) was 71%. The weights and concentrations of CoI referred to are calculated as pure material unless otherwise stated. Pregnanediol dihemisuccinate (PDHS) was prepared as already described (Grant & Marrian, 1950). The difficulty previously experienced with PDHS tending to come out of solution was not found with later batches of this compound

using the more concentrated phosphate buffer described below.

The standard medium used throughout this investigation was as follows (final concentrations): 0.114M-NaCl, 0.046M-KCl, 0.001M-MgSO₄·7H₂O and 0.056M-potassium phosphate; pH 7.4. This medium, which will be referred to as the 'saline', differs from the calcium-free phosphate saline of Krebs & Eggleston (1940) used hitherto in the higher concentration of phosphate buffer. This increase in concentration was found desirable to maintain the pH during incubation with the concentration of homogenate used. No significant difference in results was observed when Na⁺ in the saline was replaced by K⁺ in order to obtain a medium more truly representative of intracellular fluid. As alteration in the concentration of K⁺ appears to have a slightly adverse effect on the solubility of PDHS in saline, the Krebs-Eggleston type saline was retained although it admittedly does not resemble the inorganic pattern of intracellular fluid.

Homogenates were prepared from the livers of well fed female or male rats of the Wistar strain, approx. 1 year old. It was demonstrated in separate experiments (unpublished) that the sex of the animal did not influence the results. The rats were killed by dislocation of vertebrae in the neck. The livers were rapidly removed and chilled in crushed ice. After chopping roughly with scissors 3.5 g. liver were weighed into glass homogenizers (Potter & Elvehjem, 1936) standing in crushed ice, and ground with 6 ml. ice-cold water (unless otherwise stated) for approx. 1 min. with four passages of the pestle. The final homogenate was diluted to 10 ml. and kept at 0° until pipetted into incubation flasks.

The standard reaction mixture for 'incubation' experiments consisted of 2.5 ml. saline containing about 1 mg. pregnanediol as PDHS, and 1 ml. homogenate (giving a final concentration of 10% (wet wt./vol.) tissue). Unless otherwise stated, additions to this reaction mixture were made by weighing the substances concerned on a torsion balance, and adding the solid directly to the incubation flask. Freshly distilled pyruvic acid was diluted to suitable concentration, and carefully neutralized before adding to the saline.

In the quantitative experiments the procedure already described (Grant & Marrian, 1950) was followed. Incubations were in O₂ for 2 hr. at 37°. For 'controls' incubated reaction mixtures containing no PDHS were poured into 2.5 ml. PDHS saline and immediately worked up. 'Blank' values were found to be invariably low and very consistent. They were therefore not determined as a routine,

but were checked occasionally. A mean 'blank' value of 0.025 mg. 'apparent pregnanediol' was used to correct the values found in 'incubation' and 'control' experiments.

Decreased metabolism of pregnanediol observed when using rat-liver homogenates

The results of typical experiments using rat-liver homogenates are shown in Fig. 1 (bars A and B). The lower activity of liver homogenates in metabolizing pregnanediol will be evident from the fact that with these preparations over 80% of the pregnanediol originally present was recovered, as

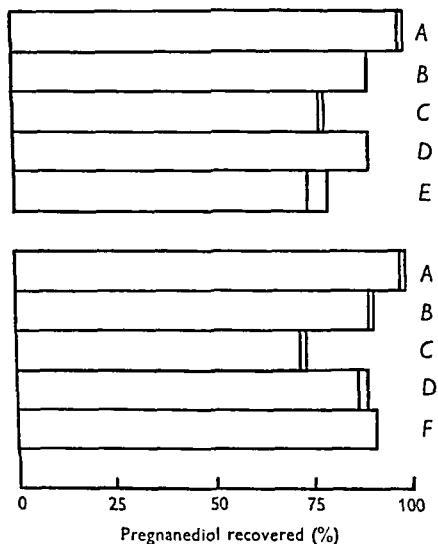


Fig. 1. Incubation of rat-liver homogenate with PDHS. Reaction mixture (RM) = about 1.6 mg. PDHS in 2.5 ml. saline + 1 ml. homogenate. Pregnanediol recovered, A, from 'controls'; after incubation of the following: B, RM; C, RM + 8×10^{-3} M-nicotinamide; D, RM using homogenate pre-incubated 0.5 hr.; E, RM using homogenate pre-incubated 0.5 hr. with added nicotineamide (final concn. 8×10^{-3} M); F, as E but nicotineamide added to homogenate after 0.5 hr. pre-incubation without nicotineamide.

In all figures two lines at ends of bars indicate two values, single lines duplicates.

compared with the recovery of only 50–60% pregnanediol incubated under the same conditions with rat-liver slices (Grant & Marrian, 1950). A similar loss of activity on mincing liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff & Sarrett, 1950) and with testosterone (Sweet & Samuels, 1948), and has been attributed by these workers to the rapid destruction of CoI in preparations of broken cells from liver and other organs (Mann & Quastel, 1941). The pH of 7.4 used in the various liver-steroid metabolism experiments reported by others and used in the present investigation is close to the maxima of 7.2 (Spaulding

& Graham, 1947) and 7.5 (Handler & Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of CoI. This destruction is, however, inhibited by nicotineamide (Mann & Quastel, 1941; Handler & Klein, 1942).

Effect of preparing homogenates in nicotineamide. As it seemed likely that the reduced activity of rat-liver homogenates could be accounted for by the destruction of CoI in the homogenate, attempts were made to protect the CoI originally present in the liver by adding nicotineamide. Homogenates were prepared for each experiment from a single rat liver as follows: (a) in saline; (b) in saline containing nicotineamide sufficient to provide a concentration of 0.2% in the final reaction mixture; (c) a portion of homogenate (a) was incubated 0.5 hr. at 37°, solid nicotineamide was then added to provide a final concentration of 0.2% (w/v) before use in PDHS experiments; (d) a portion of homogenate (b) was also incubated 0.5 hr. at 37° before use in the PDHS experiments. The results of experiments with these homogenates are shown in Fig. 1. It may be observed that there is an increase in metabolism of pregnanediol when using homogenates prepared in nicotineamide (bar C). Nicotineamide itself is not responsible for this increase since it has no effect when added to the homogenates which have been pre-incubated for 0.5 hr. (bar F). Homogenates which have been prepared in nicotineamide retain their higher activity on 0.5 hr. pre-incubation (bar E) supporting the view that the nicotineamide 'protects' the CoI originally present. The observation that 0.5 hr. pre-incubation of homogenates prepared without nicotineamide did not further decrease their activity in metabolizing pregnanediol (bar D) may find an explanation in the very rapid destruction of CoI, possibly before the pre-incubation started. Alternatively, if several pathways exist for the metabolism of pregnanediol by liver preparations and one or more is not CoI sensitive, it is conceivable that a limited direction of pregnanediol into such routes might mask the loss of activity due to decreasing concentration of CoI.

Effect of added CoI. The effect of various concentrations of added CoI on the metabolism of pregnanediol by rat-liver homogenates prepared in nicotineamide solution is shown in Fig. 2. From these results it appeared that rather high concentrations of CoI would be required to obtain the 50% metabolism previously observed with liver slices. This might be due to stimulation of metabolism by an impurity in the CoI preparation, rather than by the CoI itself. Alternatively, the concentration of nicotineamide used may have been inadequate to protect the added CoI. AMP is the chief impurity in the CoI preparation (Le Page, 1949), but when this substance in concentration of 4×10^{-3} M replaced CoI in the reaction mixture it had no effect on the

activity of the homogenate in metabolizing pregnanediol. In order to investigate the second possibility varying quantities of nicotinamide were

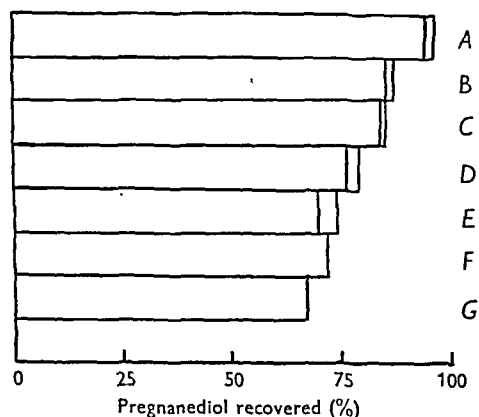


Fig. 2. Incubation of rat-liver homogenate with PDHS; influence of CoI on pregnanediol metabolism. Reaction mixture (RM) = about 1.6 mg. PDHS in 2.5 ml. saline + 1 ml. homogenate + 8×10^{-3} M-nicotinamide. Pregnanediol recovered is shown as follows: A, from 'controls'; B, after incubation of RM; after incubation of RM with CoI; C, 7×10^{-4} M-CoI; D, 1.3×10^{-3} M-CoI; E, 2×10^{-3} M-CoI; F, 2.6×10^{-3} M-CoI; G, 3.2×10^{-3} M-CoI.

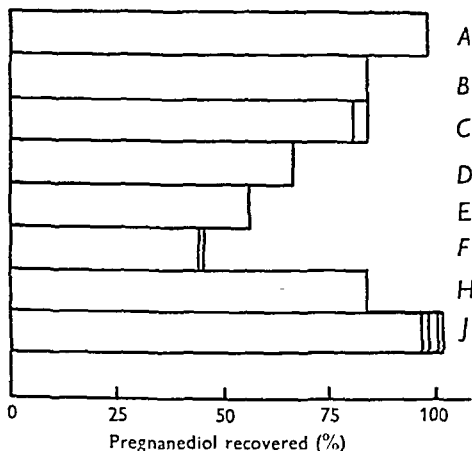


Fig. 3. Incubation of rat-liver homogenate with PDHS; protection of added CoI by nicotinamide. Reaction mixture (RM) = about 1.6 mg. PDHS in 2.5 ml. saline + 1 ml. homogenate. Pregnanediol recovered is shown as follows: A, from 'controls'; after incubation of the following: B, RM; C, RM + 8×10^{-3} M-nicotinamide; D, RM + 8×10^{-3} M-nicotinamide + 1.5×10^{-3} M-CoI; E, RM + 4×10^{-2} M-nicotinamide + 1.5×10^{-3} M-CoI; F, RM + 8×10^{-2} M-nicotinamide + 1.5×10^{-3} M-CoI; G, RM + 0.12 M-nicotinamide + 1.5×10^{-3} M-CoI; H, RM + 8×10^{-2} M-nicotinamide; J, RM without homogenate + 8×10^{-2} M-nicotinamide + 1.5×10^{-3} M-CoI.

added to the standard reaction mixture. When required, CoI was added (after the nicotinamide) to give a final concentration of 1.5×10^{-3} M. It may be seen from the results shown in Fig. 3 that an

optimum effect was obtained using 1.5×10^{-3} M-CoI and 8×10^{-2} M-nicotinamide (bar F).

This amount of nicotinamide had no significant effect in the absence of CoI (bar H). Incubation of PDHS with CoI and nicotinamide without liver homogenate did not result in any disappearance of pregnanediol (bar J).

Effect of other added substances. The following substances were tested individually in the standard reaction mixture containing 1.6×10^{-2} M-nicotinamide and were found to be without effect on the metabolism of pregnanediol-ascorbic acid (7×10^{-3} M), glutathione (3×10^{-3} M), cocarboxylase (3×10^{-4} M), riboflavin (4×10^{-4} M), pyruvate (10^{-2} M), citrate (10^{-2} M), glucose (2×10^{-2} M), ATP (10^{-2} M).

The addition of 0.025 M-semicarbazide in an attempt to trap ketonic metabolic products was also without effect.

Investigation of the products of metabolism

Experiments on a larger scale were arranged to permit the attempts to isolate the product or products of metabolism of pregnanediol by rat-liver homogenates.

For this purpose pregnanediol was purified before conversion to PDHS by recrystallization from benzene and ethanol yielding a product which melted at 237–238°. (All melting points are corrected, and unless otherwise stated were determined on a Kofler-type apparatus; Klyne & Rankeillor, 1947.) The PDHS prepared in the usual way (Grant & Marrian, 1950) from this pregnanediol melted at 147–148°. (Found (material dried at 80° for 1 hr. *in vacuo* over P_2O_5): C, 67.1; H, 8.5. Calc. for $C_{25}H_{44}O_8$: C, 67.2; H, 8.5%.)

The rat-liver homogenates were prepared in small batches in chilled glass homogenizers as described above. The Atomix Blender, with high-speed rotating blades, was not used for the preparation of the larger quantities of homogenate required since its use results in excessive frothing, the temperature is difficult to control and it has been reported by Stern & Bird (1949) and Lambden (1950) that the similar Waring Blender is capable of inactivating certain enzymes.

The large scale experiment was conducted in five parts, a total of 500 mg. pregnanediol as PDHS being incubated with rat-liver homogenate.

The following is a description of a typical experiment. PDHS (163 mg. containing 100 mg. pregnanediol) was dissolved in 245 ml. saline containing 3.5 g. nicotinamide and 0.5 g. CoI. After checking that the pH was 7.4 the solution was diluted to 250 ml. Two 2.5 ml. volumes were removed for pregnanediol analyses, the remainder was cooled in ice and water.

Fresh rat liver (70 g.) was minced roughly with scissors, mixed and divided into two parts. One part was homogenized at 0° in seven 5 g. portions with seven 15 ml. volumes of the chilled PDHS saline. These homogenate reaction mixtures were pooled, thoroughly mixed with the bulk of the PDHS saline and distributed in ten 100 ml. conical flasks. The flasks were filled with O_2 , stoppered, and shaken in a bath for 2 hr. at 37°.

The remaining 35 g. liver were treated in an identical fashion using saline which contained no PDHS and no CoI to provide a liver control experiment.

After incubation, the contents of the flasks were pooled, diluted to 300 ml. and two 3-0 ml. volumes were removed for pregnanediol analyses. The bulk of the mixture was poured into 1500 ml. cold dry acetone, thoroughly mixed in an Atomix Blender and filtered. The filter cake was washed with two 250 ml. volumes of acetone using the blender for efficient mixing. The filtrate was distilled under reduced pressure until the volume was 190 ml. The pH was found to be 7.0. This aqueous residue was transferred to large continuous liquid/liquid extractors with 50 ml. hot ethanol and

and 47%. These figures indicate the extent of metabolism in each experiment.

The purity of the pregnanediol used in these experiments was checked in a pregnanediol control experiment as follows: 70 g. rat liver were homogenized and incubated in saline without PDHS, and an ether-soluble neutral fraction was prepared from the incubated material as already described. 200 mg. pregnanediol were then added and the mixture was subjected to the Girard separations and other procedures applied in the PDHS experiments.

The pooled ketonic and non-ketonic fractions from all experiments were brown gums. The weights of the various fractions are shown in Table 1.

Table 1. *Weights of non-ketonic and ketonic fractions isolated from large scale rat-liver homogenate experiments*

	Liver incubated (g.)	Pregnanediol incubated (g.)	Fractions isolated	
			Non-ketonic (g.)	Ketonic (g.)
PDHS experiment	175	0.5	4.175	0.208
Liver control	175	0	3.760	0.132
Pregnanediol control	70	0.2	0.964	0.074

extracted with ether (250 ml.) for 6 hr. adding 10 ml. ethanol to the aqueous phase after 2 and 4 hr. The pale-yellow ether extracts were washed twice with 50 ml. volumes 0.1 N-HCl, with 50 ml. 5% (w/v) NaHCO₃ and finally with 2 × 50 ml. water. Emulsions formed during the early washings were broken by the addition of solid NaCl. The ether was evaporated to give 1.3 g. dark-brown gum in the PDHS experiment and 1.0 g. in the liver control experiment. Little significance was attached to these weights, since the gums appeared to retain solvent which was lost slowly in the vacuum desiccator. For the separation of ketonic material the ether-soluble neutral fractions, dried in a vacuum desiccator over CaCl₂ for 24 hr., were heated under reflux condenser for 1 hr. on a boiling-water bath with 0.5 g. Girard's Reagent T (Girard & Sandulesco, 1936), 1.5 g. glacial acetic acid and 10 ml. 95% ethanol. After cooling, 88 ml. water, 30 g. crushed ice and 11.5 ml. 2N-NaOH were added and the whole was extracted with 100 ml. and three 50 ml. volumes of ether. Troublesome emulsions usually formed during this extraction. The combined ether extracts were washed with 25 ml. 5% NaHCO₃ and twice with 25 ml. water. A small amount of white solid which separated at the interface between ether and water during the washing was added to the ether phase. The washed ether was taken to dryness (non-ketonic fraction). 16 ml. conc. HCl were added to the ether-washed aqueous phase to hydrolyse Girard complexes. After standing overnight ketones were extracted with 100 ml. and three 50 ml. volumes of ether. The combined ether extracts were washed and evaporated as before to give the ketonic fraction.

On account of the troublesome emulsions formed during extraction of the non-ketonic fraction in all experiments it was thought that this fraction was likely to have retained ketonic material. It was therefore subjected to a second Girard separation in each case. Ketonic and non-ketonic fractions were subsequently pooled for further examination.

The quantities of pregnanediol recovered after incubation in the five experiments, calculated as percentages of the amount of pregnanediol originally present, were 44, 48, 49, 59

and 47%. Each ketonic fraction was subjected to one further Girard separation yielding final ketonic fractions as follows: PDHS experiment, 57 mg.; liver control experiment, 13 mg.; pregnanediol control experiment, 12 mg.

Pregnan-3 α -ol-20-one from the ketonic products of metabolism of pregnanediol by rat-liver homogenates

The final ketonic fraction in each case was dissolved in 10 ml. hexane and poured on to a column of 5 mm. diameter packed with 1 g. Al₂O₃ (Peter Spence and Co., Widnes, Type H, dried *in vacuo* at 100°, Activity II (Brockmann & Schodder, 1941)). The columns were eluted successively with four or five 10 ml. portions of each of the following anhydrous solvents: hexane, benzene, ether and acetone.

In the case of the ketonic fraction isolated from the PDHS experiment crystalline residues were obtained by evaporation of the benzene eluates (26 mg.). No crystalline material was obtained in the case of the liver control or pregnanediol control experiments.

An unsuccessful attempt was made to prepare a solid semicarbazone derivative from the gum (9 mg.) eluted by hexane in the case of the PDHS experiment.

The crystalline material from the benzene eluates (PDHS experiment) dissolved in 10 ml. hexane leaving a small amount of insoluble gum. The hexane solution was poured on to a column of Al₂O₃ prepared as already described, and the column was eluted successively with four 10 ml. portions of hexane, twenty-two 10 ml. portions of benzene, six 10 ml. portions of 3:1 benzene:ether and, finally, six 10 ml. portions of ether. Evaporation of the benzene eluates gave 21.5 mg. white crystalline solid which melted at 148–150°.

After recrystallization from hexane and drying at 80° for 24 hr. *in vacuo* over P₂O₅ the substance melted at 148–149°. Mixed with authentic pregnan-3 α -ol-20-one (m.p. 147–149°) the melting point was 147–148°. $[\alpha]_D^{25} = +103.6^\circ \pm 0.5$ in ethanol ($c=0.965$). Marrian & Gough (1946) reported $[\alpha]_D^{25} = +107^\circ$ in ethanol for authentic pregnan-3 α -ol-20-one.

By treatment with semicarbazide hydrochloride and sodium acetate for 3 days at room temperature in aqueous

ethanolic solution, a product was obtained which after one crystallization from absolute ethanol and one from 80% (v/v) aqueous ethanol melted at 249–251°; mixed with authentic pregnan-3 α -ol-20-one semicarbazone (m.p. 248–250°) the melting point was 249–250°.

Investigation of non-ketonic fractions

Hexane-insoluble material was prepared from non-ketonic fractions by repeated leaching with boiling hexane and chilling the leachings for several hours in the refrigerator. In this way the non-ketonic fraction from the PDHS experiment and from the pregnanediol control experiment gave material which after recrystallization was identified as pregnane-3 α :20 α -diol. Crystalline pregnanediol (62 mg.) was obtained from the former experiment, 42 mg. from the latter. No hexane-insoluble material was obtained from the liver control experiment.

Hexane-soluble material in each experiment was dissolved in 50 ml. hexane and poured on to columns of 25 mm. diameter packed with 50 g. Al_2O_3 . The columns were eluted successively with the following solvents: hexane, benzene, ether, acetone, ethanol and methanol. Cholesterol was isolated and identified from the acetone eluates from all experiments. The gums remaining after evaporation of the other solvents resisted all attempts at crystallization.

Thus no crystalline material except pregnanediol and cholesterol was obtained from the non-ketonic fractions.

DISCUSSION

The evidence presented in this paper shows that CoI is required by the enzyme system or systems involved in the *in vitro* metabolism of pregnanediol by rat liver.

In the investigation of the products of metabolism approximately half of the pregnanediol incubated with the liver was metabolized in some way. Evidence was presented in a previous paper (Grant & Marrian, 1950) to show that the pregnanediol which disappears cannot be accounted for by the formation of conjugated substances, nor can the pregnanediol which disappears be accounted for by absorption on the relatively large weight of liver tissue, since the treatment with potassium hydroxide at 100° before extraction of the pregnanediol dissolves the tissue in addition to hydrolysing any unchanged dihemisuccinate, and added preg-

nanediol has always been recovered in good yield from these experiments carried out in nitrogen in place of oxygen. In the experiments now reported a little over 10% of the pregnanediol metabolized was accounted for as crystalline ketonic material, which appeared to consist mainly of pregnan-3 α -ol-20-one. No evidence was obtained regarding the fate of the main part of the pregnanediol which disappeared.

Sweat, Samuels & Lumry (1950) have prepared an enzyme from steer liver which catalyses the oxidation of testosterone to androst-4-ene-3:17-dione. CoI was found to be necessary as a hydrogen acceptor in the system but marked reduction of the cytochrome c concentrations of the preparation did not affect its activity. The presence of this enzyme in the livers of a variety of species, including the rat, has also been demonstrated (Samuels, Sweat, Levedahl, Pottner & Helmreich, 1950). The possibility that the same enzyme may be involved in the oxidation of pregnane-3 α :20 α -diol to pregnan-3 α -ol-20-one cannot be excluded, since Samuels and his co-workers have not investigated the effect of their enzyme on 20-hydroxy steroids (Samuels, 1951). Both the 'testosterone' and the 'pregnanediol' enzyme require CoI, and both appear to be capable of functioning in systems independent of cytochrome c.

SUMMARY

1. It has been shown that coenzyme I is required by an enzyme system or systems in rat-liver homogenates, which are able to metabolize pregnane-3 α :20 α -diol.

2. In a large scale experiment 50% of the pregnane-3 α :20 α -diol incubated with rat-liver homogenate was metabolized. Of this amount 10% was recovered as crystalline pregnan-3 α -ol-20-one. No other crystalline products of the metabolism of pregnanediol were obtained.

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Enzymic Reduction of 2:4-Dinitrophenol by Rat-tissue Homogenates

By V. H. PARKER

*Medical Research Council Unit for Research in Toxicology, Serum Research Institute,
Carshalton, Surrey*

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During an investigation into the toxic properties of 3:5-dinitro-*o*-cresol (DNOC) and 2:4-dinitrophenol (DNP) the breakdown of these substances by animal tissues *in vitro* was studied. Dinitrophenol and dinitro-*o*-cresol have been studied as inhibitors of phosphorylation in various enzyme systems and have been shown to affect the metabolism of tissues *in vitro*, but little has been reported of the effect of such systems on the dinitrophenol molecule. There is some information on the metabolic products of dinitrophenol by Magne, Mayer & Plantefol (1932) who state that the excretion products in the urine after administration of dinitrophenol to man and dogs were unchanged dinitrophenol and 2-amino-4-nitrophenol. Later, Greville & Stern (1935) demonstrated that 2-amino-4-nitrophenol was the main product after dinitrophenol had been in contact with a bacterial succinic dehydrogenase system.

The present studies were undertaken in order to ascertain the possible metabolic products of DNP and DNOC, and the organs mainly responsible for the metabolic change. As three of the amino derivatives of dinitrophenol were readily obtained, most of the following experiments were done with this compound in preference to DNOC.

EXPERIMENTAL

Preparations. Rats were killed by a blow on the head and immediately afterwards their throats were cut and the bodies bled out.

Liver homogenate. The tissue was removed, weighed and homogenized in 15 ml. of 0.06M-phosphate buffer, pH 7.4, for 1 min. Phosphate buffer was then added to make the final proportions of 3 parts of buffer to 1 part of tissue. Unless otherwise stated the homogenate was used at once.

Liver extracts. These were made by grinding a known weight of liver with washed sand and phosphate buffer in a pestle and mortar. After centrifugation, the supernatant was removed and used in volumes equivalent to 2.5 g. of homogenized liver.

Liver brei. This was prepared by mincing liver in a domestic mincing machine.

Acetone-dried liver. This was prepared by homogenizing 25 g. liver in 100 ml. of ice-cold acetone. The mixture was centrifuged and the supernatant discarded. The residue was resuspended in 100 ml. cold acetone and recentrifuged. The solid residue was washed with 100 ml. cold ether. After

centrifuging, the solid residue was spread to dry on filter paper. The dried powder was used at once.

Pieces of whole liver (approximately 0.5 g.) were freeze-dried in small bottles in a centrifugal freeze-dryer.

Homogenate experiments. For the study of the enzymic process, 8 ml. of homogenate were measured into a test tube. Inhibitors or activators also prepared in buffer solution were added at this stage. To this was added 4 ml. of dinitrophenol 0.5 mg./ml. prepared in buffer solution, pH 7.4. Finally, the volume was made up to 20 ml. with buffer solution giving a final concentration of dinitrophenol of 5.4×10^{-4} M. The contents of the tube were mixed by shaking and the tube placed in a water bath at 37°. A control tube was prepared each time containing the same concentration of dinitrophenol and inhibitor or activator in a total volume of 20 ml. of buffer solution. At intervals of 0.5 hr., 0.5 ml. samples were removed with a Krogh-Keys syringe pipette and analysed for dinitrophenol or aminonitrophenol according to the requirements of the experiment. The buffer solution, pH 7.4, was 0.06M-phosphate containing 7×10^{-2} M-succinate.

Determination of DNP. Dinitrophenol was estimated by the method based on extraction of the alkali salt with methyl ethyl ketone followed by colorimetry (Parker, 1949).

Determination of aminonitrophenols. The proteins of 0.5 ml. homogenate were precipitated by the addition of 1 ml. 15% (w/v) trichloroacetic acid. The mixture was centrifuged, 1 ml. supernatant was mixed with 2 ml. water and 1 ml. 5N-HCl. The solution was then cooled in ice water and placed in a dark cabinet. This was necessary because it was found that the diazonium compounds in solution were decomposed by daylight. Diazotization was carried out by the addition of 0.5 ml. 0.2% NaNO₂ solution, mixing and leaving in the dark for 10 min. Excess HNO₂ was removed by addition of 1 ml. 1% ammonium sulphamate solution. After a further 10 min. the diazonium compound was coupled with 2 ml. 0.5% *N*-1-naphthylethylenediamine prepared in 3N-HCl. The tube was placed in a water bath at 37° and the colour developed for 30 min. At the end of this time, the optical density of the solution was measured at 550 mμ. on a Unicam D.G. spectrophotometer. Standard curves were prepared from the two isomeric aminonitrophenols.

Extraction of the ether-soluble amino compound from liver homogenates. Liver homogenate (50 g.) was prepared as above. DNP concentration used was 100 μg. DNP/ml. After incubation for 1 hr. at 37° the reaction was stopped and the protein precipitated by 15% trichloroacetic acid, cooled and centrifuged. The supernatant liquor was made just alkaline with NH₄OH and then just acid to litmus paper with acetic acid. The solution was then extracted three times with

redistilled ether. The combined ether extracts were dried and evaporated to dryness. The brownish residue was dissolved in a small amount of ethanol (1–2 ml.).

Chromatography. A chromatograph column was prepared in an all-glass apparatus using a sludge of ethanol and alumina (P. Spence and Sons Ltd. 778–780 Salisbury House, London, E.C. 2. Activated alumina, Type H). The ethanolic extract of liver homogenate was placed on the top of the column and the chromatogram developed with ethanol containing a little NH_3 (approximately 0.15 N). The rate of development was increased by the application of a small positive pressure to the top of the column. As a rule, three bands were seen moving separately down the column. The last band was eluted by changing the composition of the developing fluid to one of 90% ethanol containing 0.15 N- NH_3 . Each component was collected separately as it appeared.

Paper-partition chromatography. A pencil line was drawn across a strip of Whatman filter paper no. 3 (25 × 3.5 cm.) at a distance of 4 cm. from one end, a second pencil line at 15.0 cm. from the first. A small quantity (10–30 μl .) of the ethanolic extract of liver homogenate was placed on the lower line and dried. With each chromatogram of the unknown substance a control was run consisting of a mixture of DNP and the isomeric amino compounds. A spot of this mixture was placed on the line on one side of the 'unknown' spot. The strip was suspended in a gas jar so that the end of the paper nearest to the spots of solutions was just dipping into the developing fluid. The gas jar was filled with N_2 by displacement of air and closed with a greased lid and development of the chromatogram allowed to take place by upward capillary attraction until the liquid front reached the second pencil line. At this stage the paper was removed and allowed to dry in the air. The developing fluid consisted of 20% benzene, 20% pentanol, 40% ethanol and 20% ammonia solution (sp.gr. 0.880). All percentages were v/v.

Spectroscopy. Some of the solutions obtained by chromatography were examined for ultraviolet absorption in the Unicam Quartz S.P. 500 spectrophotometer. The absorption curves were determined from 250 to 400 $\text{m}\mu$. at a slit width of 0.1 mm.

All but two of the following experiments took place in buffered solutions at pH 7.4. The conditions for the two exceptions are mentioned in the text.

RESULTS

Characteristics of the enzyme preparation. The activity of the homogenates, measured as μg . DNP destroyed per ml. of solution in 1 hr. was at a maximum when the liver was prepared in phosphate buffers at pH values of 7.0–7.5.

The optimum temperature was approximately 37°. Heating the homogenate to 100° resulted in complete loss of activity, whilst maintaining the preparation at 60° for 10 min. resulted in a decrease in activity by 70%. There was no destruction of DNP when the homogenate was kept at 0°.

The activity of the preparation depended upon the length of time taken to homogenize the liver and upon the age of the homogenate. Preparations homogenized for periods of time varying from

30 sec. to 6 min. exhibited varying degrees of activity, the most active preparation being that which had been homogenized for 1 min. Experiments also showed that the homogenate was most active if used immediately after preparation. Left for periods of up to 2 hr. at 37° the homogenates lost 20% of their activity. From 2 to 4 hr. there was little further loss in activity.

Michaelis constant, K_m and effect of enzyme concentration. In order to determine the Michaelis constant for the reaction, it was necessary to remove samples from the homogenate at intervals of 5 min. The reciprocals of the amount of DNP destroyed in 5 min. ($1/v$) were plotted against the reciprocals of the concentration ($1/c$) as suggested by Lineweaver & Burk (1934). The Michaelis constant was derived from the slope of the line, K_m/v_m , and it was found to be $8.5 \times 10^{-5} \text{ M-DNP}$ (Fig. 1).

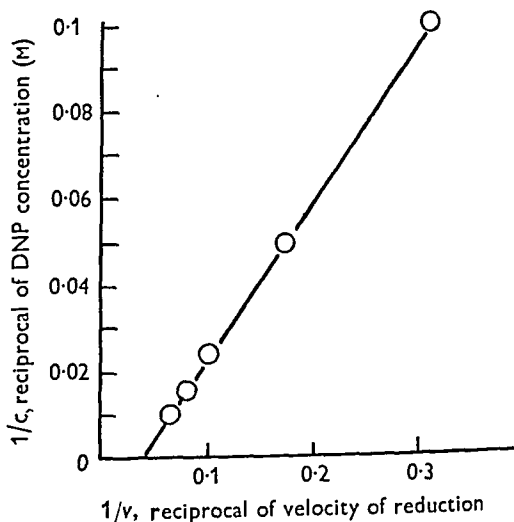


Fig. 1. Effect of DNP concentration within the range of 5.4×10^{-5} to $5.4 \times 10^{-4} \text{ M}$ on the amount of DNP destroyed by liver homogenates. v = moles DNP destroyed in 5 min.

Fig. 2 shows the result of varying the concentration of the homogenate. There is a linear relationship between the amount of enzyme present and the amount of DNP destroyed when not more than 80% of DNP is destroyed.

Comparison of homogenates with other preparations. When the activities of liver extract and liver brei were compared with the activity of an equivalent quantity of liver homogenate it was found that under identical conditions there was no difference between the three forms of preparations. In other experiments, acetone-dried liver and freeze-dried liver were compared with homogenates. Acetone-dried liver powder equivalent to 4 g. of fresh liver destroyed only 30% of DNP (initial concn. $5.4 \times 10^{-4} \text{ M}$) in 1 hr. compared with 60% of DNP destroyed by 2.5 g. of homogenized liver in the same time. Whole liver freeze-dried had approximately

90 % of the activity of homogenized liver. Freeze-dried liver retained its initial activity in a refrigerator for two weeks.

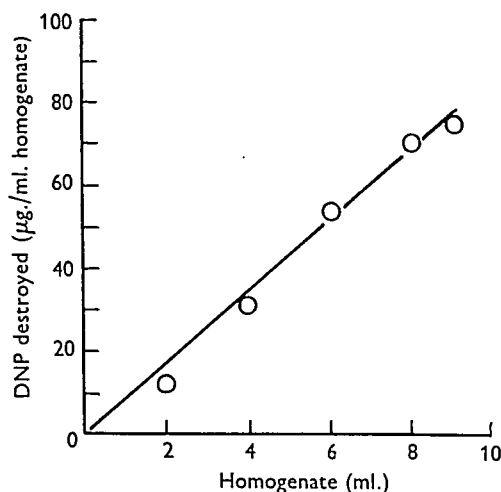


Fig. 2. Effect of enzyme concentration on amount of DNP destroyed. Enzyme concentration is expressed as ml. of liver homogenate in range of 2–9 ml. (15 g. liver homogenized and made up to a final volume of 60 ml.). Each solution analysed after 1 hr. at 37°. Initial DNP concentration $5.4 \times 10^{-4}M$.

An attempt was made to determine the active portion of the homogenate. An homogenate was divided into three portions *A*, *B* and *C*. *A* was incubated with DNP in the usual way. *B* was centrifuged

Table 1. Activity of liver homogenate fractions

(Preparation *A*, 2.5 g. liver homogenized, DNP added and volume adjusted to 20 ml. Preparation *B*, 2.5 g. liver homogenized in 10 ml. buffer solution, centrifuged, DNP added to supernatant fluid. Volume adjusted to 20 ml. Solid residue from *B* suspended in buffer solution, DNP added and volume adjusted to 20 ml. Preparation *C*, 2.5 g. liver homogenized in 10 ml. of buffer solution, centrifuged and supernatant fluid filtered through kieselguhr. Filtrate adjusted to 20 ml. after addition of DNP. DNP concentration, 100 µg./ml., pH 7.4.)

Preparation	Whole homogenate of <i>A</i>	Supernatant of <i>B</i>	Residue of <i>B</i>	Filtered supernatant of <i>C</i>
µg. DNP destroyed per ml. after 60 min.	56	41	13	8
Activity expressed as percentage activity of whole homogenate	100	73	23	15

at the top speed of an angle centrifuge and the supernatant fluid and solid residue incubated separately with DNP. *C* was also centrifuged, but the supernatant fluid was filtered through a layer of kieselguhr before assessment of its activity. Table 1 indicates that most of the activity resides in the

opalescent supernatant fluid after centrifuging, but is lost by filtration.

Effect of enzyme inhibitors. The following compounds were examined for their effect on the activity of liver homogenates: potassium cyanide, *p*-chloromercuribenzoic acid (CMBA), iodoacetic acid, sodium pyrophosphate, sodium azide and sodium malonate. Table 2 shows that only cyanide inhibited the destruction of dinitrophenol.

Table 2. Effect of inhibitors on destruction of DNP

(For each test, 2.5 g. of liver were homogenized in 15 ml. of phosphate buffer, and 1 ml. of inhibitor added. After incubation at 37° for 30 min., DNP was added to a final concentration of $5.4 \times 10^{-4}M$ and a total volume of 20 ml. Samples were removed for analysis at 30 and 60 min.)

Compound	Final concn. (M)	Inhibition (%)
Potassium cyanide	5×10^{-5}	32
	5×10^{-4}	90
	5×10^{-3}	100
Sodium azide	5×10^{-3}	8
Iodoacetic acid	5×10^{-3}	8
<i>p</i> -Chloromercuribenzoic acid	5×10^{-3}	0
Sodium pyrophosphate	5×10^{-2}	0
Sodium malonate	5×10^{-3}	0

Other factors influencing the breakdown of DNP. Greville & Stern (1935) and Westfall (1943) used a dehydrogenase system to demonstrate the reduction of nitro groups in dinitrophenol and trinitrotoluene. The effect of substrates for dehydrogenases was observed on the activity of liver homogenates in destroying DNP. It was found that their activity could be increased by almost 20 % when $7 \times 10^{-2}M$ -succinate or lactate was added. The experiments reported above were carried out under aerobic conditions. Westfall demonstrated that the reduction of trinitrotoluene also occurred under anaerobic conditions. The rate of decomposition of DNP under aerobic and anaerobic conditions were compared. DNP was added to chilled liver homogenate in Thunberg tubes immersed in ice water. The DNP concentration was $5.4 \times 10^{-4}M$ after adjustment to a known volume with phosphate buffer, pH 7.4. The tubes were evacuated and refilled with nitrogen three times in succession. These tubes as well as similar tubes, open to the atmosphere, containing the same amount of liver homogenate and DNP were incubated at 37° for 1 hr. It was found that the reaction took place at the same rate under aerobic and anaerobic conditions.

That the succinic oxidase system was not responsible for the reduction of dinitrophenol was demonstrated by the following experiment. Succinic oxidase was prepared by Slater's modification of Keilin & Hartree's method (Slater, 1949). The final preparation was such that 0.5 ml. of a 1 in 25 dilution

Table 3. *Enzymic activity of rat and rabbit tissues*

(Spleen, kidney, heart, intrascapular fat and brain tissues were removed from the freshly killed animal, weighed and homogenized in 3 parts of phosphate buffer. Liver and muscle, 2-3 g. weighed and homogenized in 3 parts of phosphate buffer. DNP added to a final concentration of 5.4×10^{-4} M. Blood, 5 ml. taken, DNP in phosphate added to a final concentration of 5.4×10^{-4} M-DNP. All homogenates incubated at 37° and analysed after 30 min. DNP destroyed calculated in terms of mg./100 g. wet wt. tissue in 30 min.)

	Rat tissue		Rabbit tissue	
	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)
Liver	68	100	29	100
Spleen	40	59	0	0
Kidney	41	60	12	41
Heart	20	29	1	3
Intrascapular fat	33	47	—	—
Muscle	11	16	—	—
Brain	2	3	—	—
Blood	0	0	0	0

reduced 1 ml. 10^{-5} M-methylene blue in 30 min. For the determination of its effect on DNP 1 ml. of the undiluted enzyme preparation was used. The buffer solution, pH 7.7, was 0.01 M-phosphate solution containing 0.03 M-sodium bicarbonate. The Thunberg tubes were filled with 5% carbon dioxide in nitrogen after removal of air. It was found that with DNP concentrations of 4.5×10^{-5} to 4.5×10^{-4} M no destruction of the nitro compound occurred.

Distribution of the enzymic activity. The activity of other tissues of the rat was examined by making homogenates from them and incubating with dinitrophenol under identical conditions.

Table 3 shows that the activity of the rabbit tissue is lower than that of the corresponding rat tissue. On the other hand, when injected into the living animal, DNP disappears from the blood stream of the rabbit within a few hours, whereas it remains in the blood of the rat for several days (Parker, Barnes & Denz, 1951).

Reduction of DNOC. Experiments were performed with rat homogenates in which DNOC was substituted for DNP. It was found that these homogenates could reduce DNOC and the rate of reduction was measured as rate of disappearance of DNOC. Various tissues had similar relative capacities of reducing DNOC as they had in reducing DNP. The product of DNOC was a diazotizable amine, but was not identified.

The reduction product of DNP

The experiments described above indicated that DNP was destroyed by liver homogenates by an enzymic process. The products of this reaction were sought. A homogenate which had been in contact with DNP for 1 hr. was taken. The reaction was stopped by addition of 5 ml. of 20% trichloroacetic acid to every 20 ml. of homogenate. A portion of the supernatant was diazotized and coupled with *N*-1-naphthylethylenediamine. A magenta dye

was produced. On the assumption that only primary aromatic amines form dyes of this kind, it was decided that at least one of the metabolic products was such an amine. The possible amino derivatives of 2:4-dinitrophenol are 4-amino-2-nitrophenol, 2-amino-4-nitrophenol and 2:4-diaminophenol. The isomeric nitroamines can be separated from the diamine by simple ether extraction in acid solutions, and it was found that the greater part of the amino fraction could be extracted in this way.

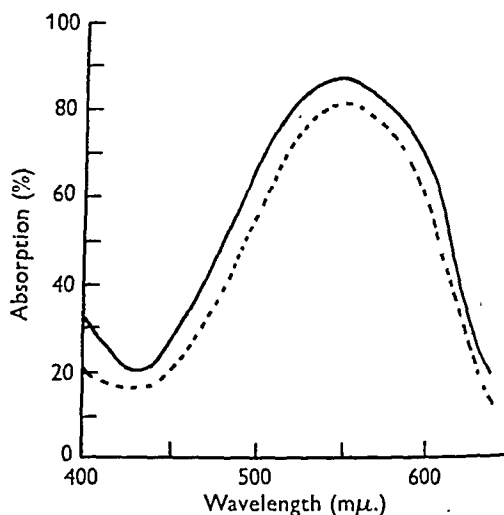


Fig. 3. Absorption curves of diazo dyes of aminonitrophenols. Aminonitrophenol diazotized (25 μ g.) and coupled with *N*-1-naphthylethylenediamine in a final volume of 7.5 ml. —, dye of 2-amino-4-nitrophenol; - - -, dye of 4-amino-2-nitrophenol.

The diazotization method could be used to estimate either of the two aminonitrophenols separately. But the diazo dyes that were produced did not differ sufficiently to enable one amine to be detected in the presence of the other. The characteristics of the absorption curves are given in Fig. 3.

It is seen that both dyes have very nearly the same intensity of colour and the maximum absorption occurs at the same wavelength. Magne *et al.* (1932) had detected 2-amino-4-nitrophenol in the freshly voided urine of workers exposed to DNP dust. Their method was a modification of the Derrien test in which the solution was diazotized, coupled with alkaline 2-naphthol and the resultant dye extracted into ether. After addition of acetic acid and zinc ions to the ethereal solution, the 2-amino-4-nitrophenol was identified by the production of a pink colour. Under the same conditions the 4-amino-2-nitro compound produced a yellow colour. The authors quoted above used this method for the examination of the liver of a dog which had been poisoned by DNP, and in this case they concluded that 4-amino-2-nitrophenol was present.

When the Derrien test was used to examine liver homogenates, a yellow colour was produced. This was an indication of the presence of 4-amino-2-nitrophenol, but was not conclusive evidence for the absence of the isomer. Excess of 4-amino-2-nitrophenol could hide the presence of smaller amounts of 2-amino-4-nitrophenol.

A mixture of the pure compounds was added to an alumina column. On development with ammoniated ethanol, a yellow band first appeared which very soon moved well ahead of the bulk of the coloured compounds remaining at the top of the column. This yellow compound was eluted and identified as DNP. The two aminonitrophenols did not separate completely. The 4-amino isomer moved rather faster than the 2-amino compound but became diffuse. The result was a broad band which was pink at the bottom and orange at the top, the pink compound being 4-amino-2-nitrophenol. However, by paper-partition chromatography, all three compounds could be separated. The R_F of DNP was 0.60–0.65, for 2-amino-4-nitrophenol it was 0.42–0.44 and for 4-amino-2-nitrophenol it was 0.30–0.32. For the purpose of identifying and isolating the metabolic product of DNP, the two chromatographic methods were combined. An ethanolic extract of liver homogenate after incubation with DNP was added to an alumina column. A yellow band of unchanged DNP appeared and was eluted. This was followed by a pink band. Finally, after elution of the pink band, there remained at the top of the column an orange band which could only be removed after increasing the water content of the developing fluid to about 20% and it was found that this fluid did not contain a diazotizable amine.

The pink fraction obtained from the alumina column produced an orange solution after elution. This solution was evaporated to dryness in an atmosphere of nitrogen and the dry residue dissolved in 2–4 ml. of ethanol. Approximately 20 μ l. of this

solution was placed upon a paper strip and developed. After completion of the chromatogram three spots were seen: a yellow spot of the same R_F value as DNP; a brownish pink spot of R_F value of 4-amino-2-nitrophenol; and a faint orange spot at the position of 2-amino-4-nitrophenol. Subsequent analysis after cutting the spots from the paper revealed that 90% of the total amine content of the extract was 4-amino-2-nitrophenol and the orange spot accounted for the remaining 10%.

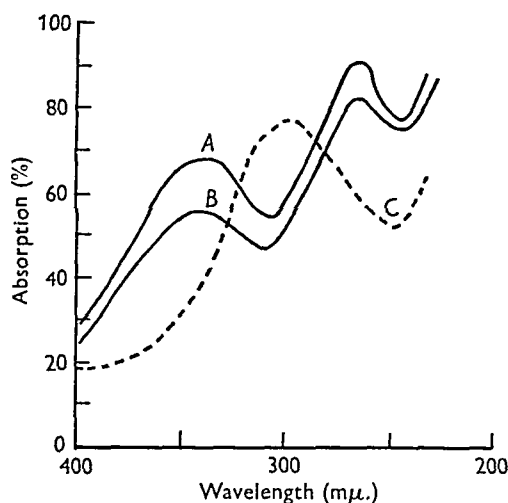


Fig. 4. Ultraviolet absorption of aminonitrophenols compared with metabolite. A, 0.0015% 4-amino-2-nitrophenol in 0.001N-HCl. B, metabolite washed from paper chromatogram with 0.001N-HCl. Estimated to contain 0.0012% of 4-amino-2-nitrophenol. C, 0.001% 2-amino-4-nitrophenol in 0.001N-HCl.

Further evidence of identity was supplied by spectrography. After preliminary separation on an alumina column and concentration into a small volume the amine fraction was placed on a paper strip and developed. The compound of the R_F value of 4-amino-2-nitrophenol was cut from the paper and dissolved in 0.001N-hydrochloric acid. Its ultraviolet absorption spectrum was observed and compared with that of 4-amino-2-nitrophenol and of 2-amino-4-nitrophenol (Fig. 4). It is seen that the absorption curve of the metabolite of DNP strongly resembles that of 4-amino-2-nitrophenol.

Thus from the evidence of chromatography and spectrography it is concluded that 4-amino-2-nitrophenol is the main reduction product of DNP in liver homogenates.

The relationship between the rate of DNP destruction and the rate of formation of amino compounds was next investigated. An experiment was made in which the amount of DNP destroyed was measured together with the amount of amine produced. The latter, calculated as 4-amino-2-nitrophenol, was determined in the trichloroacetic

acid supernatant and was thus equivalent to the total amine. It was observed that during the early stage of rapid removal of DNP there was a rapid increase in the concentration of amino compounds, but that after 3 hr. loss of amine occurred while DNP destruction continued (Fig. 5).

Earlier in this paper it has been noted that the bulk of the amino compounds were extracted by ether from acidified solutions. However, examination of the residual aqueous solution showed that some amine was still present. This compound was

At intervals samples were removed and analysed for ether-soluble amine and ether-insoluble amine. The results of these experiments are shown in Figs. 7 and 8. Both compounds were destroyed by homogenates but at different rates. The 2-amino-4-nitrophenol is slowly destroyed with a gradual accumulation of the ether-insoluble compound. 4-Amino-2-nitrophenol is more rapidly removed. In 5 hr. very little of the ether-soluble 4-amino-2-nitrophenol is left, but there is only a slight increase of the water-soluble compound.

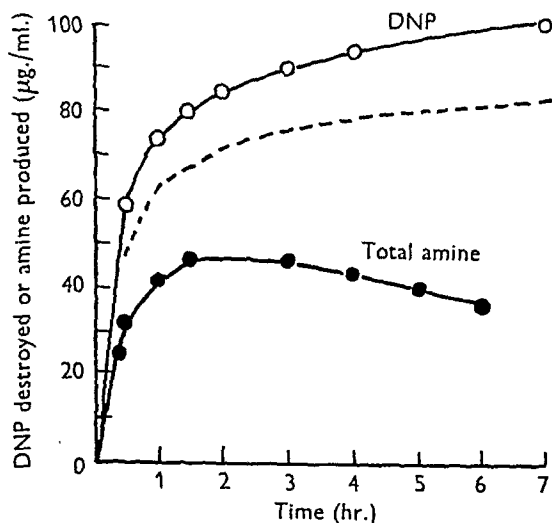


Fig. 5. Comparison between DNP destroyed and amine produced by liver homogenate. Initial DNP concentration 100 µg./ml. homogenate. Dotted line is theoretical amount of 4-amino-2-nitrophenol to be expected calculated from DNP destroyed assuming 100% recovery and no destruction of amine. ○—○, DNP destroyed; ●—●, amine produced.

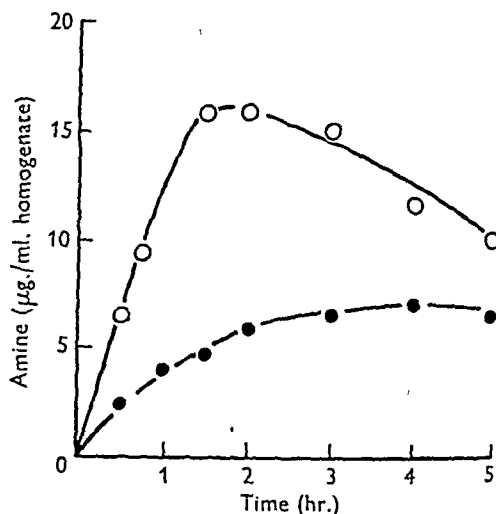


Fig. 6. Comparison between production of ether-soluble and ether-insoluble amine during course of DNP destruction. Initial concentration of DNP, 100 µg./ml. (Trichloroacetic acid supernatant fractionated and analysed as described in text.) Results read as 4-amino-2-nitrophenol. ○—○, ether-soluble amino compounds; ●—●, ether-insoluble amino compounds.

not extractable by ether, benzene or light petroleum, behaving in this respect as 2:4-diaminophenol. This fraction was termed the 'ether-insoluble' fraction. A similar experiment to that described above was performed with the additional elaboration of extracting the trichloroacetic acid solution with ether. The ether-soluble and ether-insoluble fractions were then analysed for amino compounds. Fig. 6 shows that as the DNP is destroyed the ether-soluble amine at first increases rapidly, but later decreases in concentration. At the same time there is a slow increase of the ether-insoluble amine the level of which is maintained after the concentration of the ether-soluble amine begins to fall. The decrease of the ether-soluble fraction commences after 3 hr. incubation. Thus it would appear that 4-amino-2-nitrophenol is itself destroyed.

The destruction of the two aminonitrophenols was next investigated. In turn, each of the above compounds was incubated with liver homogenates.

That the mechanism of this destruction was similar to the destruction of DNP and might possibly involve the same enzymes was demonstrated by experiment. It was found that the destruction of the amino nitro compounds could be inhibited by heat and cyanide ions but not by sodium azide, sodium malonate, *p*-chloromercuribenzoic acid or iodoacetic acid. Strict summation experiments were difficult to perform as the product of DNP reduction, 4-amino-2-nitrophenol, could not be distinguished by the method of estimation from the added amine. Moreover, the 4-amino-2-nitrophenol is itself destroyed by the liver homogenate and might also compete for the enzyme system. An indication that the presence of amino-nitrophenols lowers the rate of DNP destruction and may be competing for the enzyme system was obtained by the experiment illustrated in Fig. 9. Three quantities of homogenate were prepared. The first contained DNP, the second DNP and 2-amino-4-nitrophenol and the third DNP and 4-amino-2-nitrophenol.

Samples were taken from each homogenate at intervals of 15 min. It is seen that both compounds inhibit the destruction of DNP, but that 4-amino-2-nitrophenol has the greater effect.

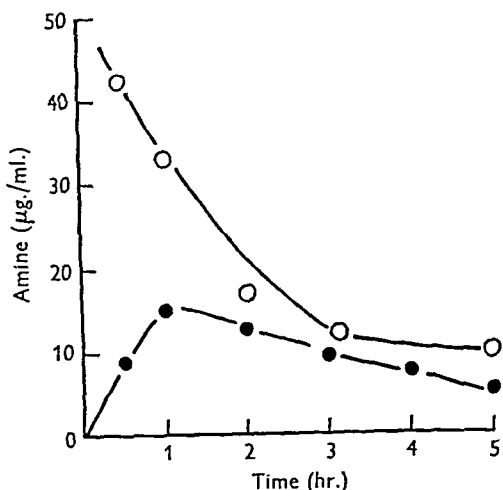


Fig. 7. Rate of destruction of 4-amino-2-nitrophenol in liver homogenate. Initial amine concentration of 100 µg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 4-amino-2-nitrophenol; ●—●, ether-insoluble amine.

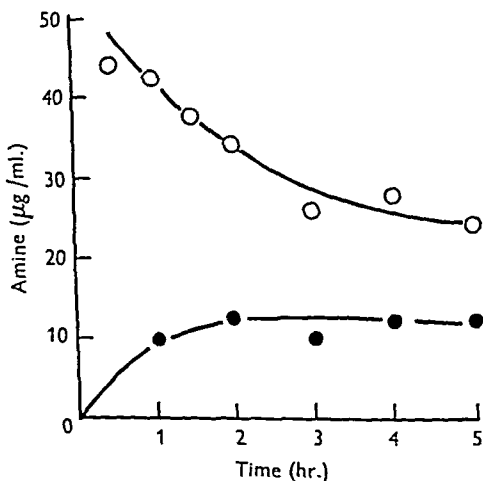


Fig. 8. Rate of destruction of 2-amino-4-nitrophenol in liver homogenate. Initial amine concentration of 100 µg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 2-amino-4-nitrophenol; ●—●, ether-insoluble amine.

It has been shown that DNP is reduced *in vitro* by animal tissues to 4-amino-2-nitrophenol with only a slight amount of 2-amino-4-nitrophenol. It was, therefore, of interest to compare this result with the urine excretion products of rats poisoned by DNP. Male rats of 200 g. weight were given 20 mg. DNP/kg. body weight by subcutaneous injection.

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The animals were kept in metabolism cages for the next 24 hr. and their urine collected. The urine was extracted in the same way as described for the homogenates. The dried extract was dissolved in a few drops of ethanol. Approximately 20 µl. of the extract was then developed on a paper chromatogram and compared with a known solution containing DNP and the two isomeric aminonitrophenols. Two spots were obtained from the urine extract. One had an R_F value identical with the spot due to pure DNP. The second spot was orange in colour and had the R_F value of 2-amino-4-nitrophenol. There was no sign of 4-amino-2-nitrophenol.

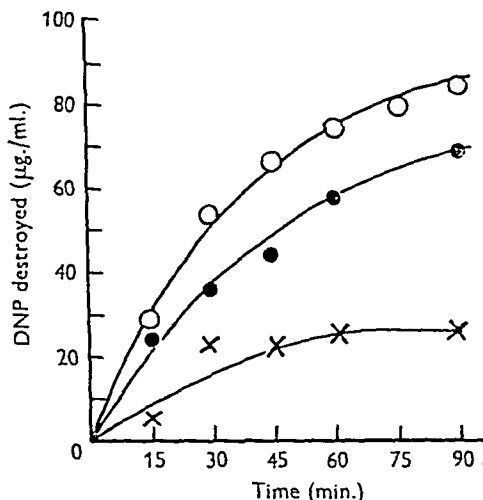


Fig. 9. Influence of aminonitrophenols on rate of destruction of DNP. ○—○, normal homogenate containing DNP at initial concentration of 100 µg./ml.; ●—●, homogenate containing DNP at 100 µg./ml. + 2-amino-4-nitrophenol at 200 µg./ml.; ×—×, homogenate containing DNP at 100 µg./ml. + 4-amino-2-nitrophenol at 200 µg./ml.

It has been shown that the latter compound is more rapidly destroyed *in vitro* than its isomer. It might be expected, therefore, that 2-amino-4-nitrophenol would appear as the predominant reduction product of DNP in the urine.

It has been remarked above that the 2-amino isomer was the compound found in the urine of men and dogs poisoned by DNP (Magne *et al.* 1932). It is of interest to note that the latter workers found 4-amino-2-nitrophenol in the liver of a DNP-poisoned dog.

DISCUSSION

The enzymic reduction of aromatic nitro groups is well known. Westfall (1943) and Bueding & Jolliffe (1946) have demonstrated that trinitrotoluene (TNT) is reduced by the succinic oxidase system to an amino derivative. The nitro group of chloromycetin is reduced by bacterial enzymes (Smith & Worrel, 1949) and has been related to a nitrite

reductase (Egami, Ebata & Sato, 1951). Greville & Stern (1935) showed that a bacterial succinic dehydrogenase would reduce 2:4-dinitrophenol. The present paper confirms that animal tissues can reduce one nitro group of DNP to an amino group. The process is similar to that described for TNT inasmuch that substrates of dehydrogenases, lactate and succinate, increase the rate of reduction. However, it was not possible to reduce DNP by a preparation of heart-muscle succinic oxidase. In this connexion, it may be noted that Bueding & Jolliffe did not obtain a reduction of TNT unless coenzyme I was added. Further work is necessary to see if there is any relation between the reduction of DNP with the respiratory activity of the tissue.

The next stage of the process, destruction of the amine, appears to be similar in properties to the first stage as both are inhibited by cyanide ions and not by the other enzyme inhibitors mentioned. It is possible that the reduction of the nitro group in the 4 position is followed by reduction of the 2-nitro group. Pure 2:4-diaminophenol is not extracted from water by organic solvents and would therefore be contained in what is here termed the 'ether-insoluble' fraction. Definite identification of this fraction as 2:4-diaminophenol has not been made.

It is interesting that the amino compound appearing in the urine of animals poisoned by DNP is the 2-amino-4-nitro compound. The experiments reported above show that this compound is destroyed less rapidly than its isomer. The excretion

products in the urine represent the balance of production and destruction of both aminonitrophenols.

SUMMARY

1. 2:4-Dinitrophenol is reduced by rat-liver homogenates to 4-amino-2-nitrophenol. Some 2-amino-4-nitrophenol is obtained (about 10% of the total amine).

2. Maximum rate of reduction of 2:4-dinitrophenol is attained in phosphate buffer of pH 7 at 37° and in the presence of sodium lactate or sodium succinate.

3. The reaction is completely inhibited by 5×10^{-3} M-potassium cyanide.

4. Rat spleen, kidney, intrascapular fat, muscle and brain also destroy 2:4-dinitrophenol.

5. The reduction products were isolated by means of alumina chromatograms and paper-partition chromatography. 4-Amino-2-nitrophenol was identified by ultraviolet spectrography.

6. Liver homogenates can reduce the isomeric aminonitrophenols. The second reduction product is thought to be 2:4-diaminophenol, but has not been identified.

7. The main excretion products of 2:4-dinitrophenol in the urine of rats were unchanged 2:4-dinitrophenol and 2-amino-4-nitrophenol.

My thanks are due to Dr S. Ellingworth, Messrs Imperial Chemical Industries Ltd., for the gift of the aminonitrophenols and to Miss A. E. Scott for valuable technical assistance.

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The Metabolism of some Saturated Aliphatic Hydrocarbons, Alcohols and Fatty Acids by *Proactinomyces opacus* Jensen (*Nocardia opaca* Waksman & Hendrik)

By D. M. WEBLEY

Macaulay Institute for Soil Research, Aberdeen

AND P. C. DE KOCK

Botany Department, The University, Aberdeen

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Erikson (1949), as a result of a study of about 300 strains of *Proactinomyces*, mostly isolated from Rothamsted soils, came to the conclusion that the partially acid-fast strains in that locality were mainly accounted for by *Proactinomyces opacus*, *Pro. salmonicolor* and *Pro. paraffinae*. All three species grow well on liquid paraffin or paraffin wax as the sole energy source.

A few papers have been published on the oxidation of aliphatic hydrocarbons by washed suspensions of pure cultures of micro-organisms. Both Johnson, Goodale & Turkevitch (1942) and Imelik (1949) used species of *Pseudomonas*. It was shown by Johnson *et al.* (1942) that aliphatic hydrocarbons ranging from C_6 to C_{12} were oxidized. Saz (1949), using four strains of pathogenic mycobacteria, found that in particular the 14-, 16- and 18-membered hydrocarbons, both saturated and unsaturated, greatly increase the oxygen uptake. Cetyl (hexadecyl) and stearyl (octadecyl) alcohols had a similar effect. A useful review on the assimilation of hydrocarbons by micro-organisms has been published by ZoBell (1950).

In this paper we have made a study of the aerobic metabolism of a strain (T16) of the non-pathogenic *Pro. opacus* (Jensen) (*Nocardia opaca* Waksman & Hendrik, *Bergey's Manual*, 6th ed.), employing the Warburg technique.

METHODS

Growth of the organism. *Pro. opacus*, strain T16, isolated by Erikson from soil, was used throughout this work.

The organism was grown in liquid culture. Glucose proved to be the most suitable carbon source for maximum yield of cells. Although good growth took place in the presence of *n*-dodecane and *n*-tetradecane, it was found impossible to separate the cells from the hydrocarbons by centrifuging. The final medium employed was of the following composition: glucose, 20 g.; $NaNO_3$, 2 g.; KH_2PO_4 , 0.5 g.; $NaCl$, 0.2 g.; $MgSO_4$, 0.2 g.; $FeCl_3$, trace; 1 l. distilled water; pH adjusted to 7.2. The medium was distributed in 500 ml. media storage bottles (200 ml./bottle) and inoculated with 8 ml. of a 48 hr. culture of the organism. Growth was

carried out at room temperature on a rotary shaking machine. After 5-6 days, the cells were harvested by centrifuging, washed once with distilled water and finally suspended in distilled water. 1 ml. of suspension was usually made to contain about 6 mg. (dry wt.) of organism. The suspension could be kept for periods up to 2-3 weeks at 2° until needed. The experiments quoted in this paper have been performed with suspensions stored for 7-14 days at 2°.

Oxygen uptake. This was measured in a Warburg apparatus at 20°, the final total volume in each vessel being 2.5 ml., made up as follows: 1 ml. suspension; 0.5 ml. 0.066M phosphate buffer, pH 7.0; substrate and distilled water.

Owing to the high endogenous respiration, particularly when newly harvested, it was usually necessary to leave the organism and phosphate buffer shaking gently in the vessels overnight. This preliminary incubation, while markedly reducing the blank, had little effect on the oxidation of the subsequently added substrates. Next morning, 0.2 ml. of 5% (w/v) KOH and fluted filter paper, for the absorption of CO_2 , were introduced into the centre cup of each vessel. Distilled water was added directly to the vessel and substrates, if liquid, were tipped in from the side arm after equilibration. In the case of some hydrocarbons and alcohols, it was found advantageous to add 0.2 ml. distilled water to the side arm simultaneously to facilitate tipping, so ensuring that all the substrate passed into the main vessel. In the case of solid substances, these were added directly just before the equilibration period of 10-15 min. For the measurement of B.Q., three manometers were used for each determination (Umbreit, Burris & Stauffer, 1948).

RESULTS

Oxygen uptake of washed suspensions in the presence of liquid aliphatic hydrocarbons

Fig. 1 gives the result of an experiment showing the effect on the oxygen uptake of suspensions of *Pro. opacus* of the addition of the hydrocarbons decane, undecane, dodecane, tetradecane and hexadecane. It is evident that the higher paraffins (dodecane, tetradecane and hexadecane) most markedly affect the oxygen uptake. Saz (1949), working with tubercle bacilli, also found that with tetradecane, hexadecane and octadecane the oxygen uptake was

greatly increased. With *Pro. opacus* it was similarly observed (Saz, 1949) that the organism tended to migrate to the hydrocarbon-water interface.

As the organism is frequently cultured on liquid paraffin, it was thought of interest to find the effect of this substance on the rate of oxygen uptake by

Oxygen uptake in the presence of solid hydrocarbons

Solid paraffins increased the oxygen uptake of *Pro. opacus*. Included in Table 2 are dodecane, tetradecane and hexadecane (liquid at 20°) for comparison.

Table 2. Oxygen uptake of *Proactinomyces opacus* in the presence of some solid aliphatic hydrocarbons

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, incubated overnight at 20°. Next morning substrates and distilled water were added to give a final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	23
Paraffin wax (0.2 g.)	56
<i>n</i> -Octadecane (0.2 g.)	95
<i>n</i> -Hexadecane (0.2 ml.)	173
<i>n</i> -Tetradecane (0.2 ml.)	82
<i>n</i> -Dodecane (0.2 ml.)	90

The effect of hydrocarbons on oxygen uptake in the presence of glucose

It will be seen from Table 3 that no stimulation of oxygen uptake occurs when glucose and hydrocarbon are present together. In fact, the sum of the separate rates is greater than when the two substrates are oxidized simultaneously. Johnson *et al.* (1942) report similar findings for *Pseudomonas* sp. when glucose and octane were added separately and together to their suspensions. These workers came to the conclusion that 'the fact that they are largely additive indicates that different dehydrogenase systems are involved'.

It is also of interest to note from Table 3 that the rate of oxygen uptake in the presence of glucose is substantially the same as for hexadecane.

Table 3. The effect of hydrocarbons on the oxygen uptake of *Proactinomyces opacus* in the presence of glucose

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning substrates and distilled water added to give a final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	27
Glucose (0.4%, w/v)	196
<i>n</i> -Dodecane (0.2 ml.)	102
Glucose (0.4%, w/v) + <i>n</i> -dodecane (0.2 ml.)	265
<i>n</i> -Hexadecane (0.2 ml.)	192
Glucose (0.4%, w/v) + <i>n</i> -hexadecane (0.2 ml.)	322

Determination of R.Q. of dodecane

The theoretical R.Q. for the complete oxidation of a hydrocarbon is 0.65. For dodecane, the value obtained was 0.72 and that for the endogenous

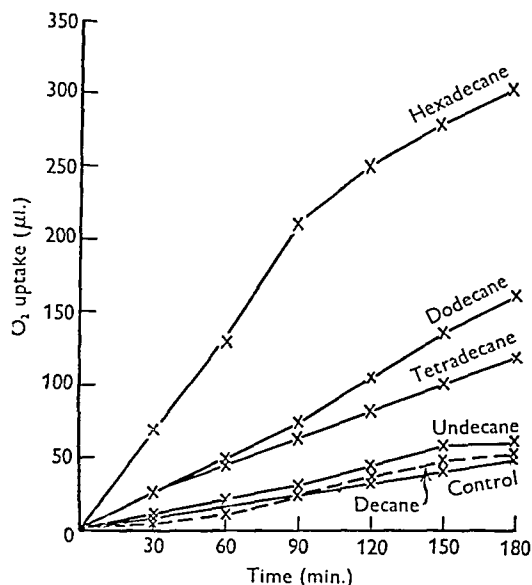


Fig. 1. The oxygen uptake of *Proc. opacus* in the presence of *n*-decane, *n*-undecane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. A washed suspension (1 ml.) of organism + 0.5 ml. phosphate buffer, pH 7.0, shaken gently overnight. Distilled water and substrates (0.2 ml. from side arm) added next morning. Temp. = 20°.

Pro. opacus. The results are given in Table 1. It appears from Table 1 that liquid paraffin is not as good a substrate as dodecane and tetradecane. Growth experiments also showed that better growth took place on dodecane and tetradecane than on liquid paraffin when these substances were used as sole carbon source in a mineral salt medium of the same composition as given under Methods (the hydrocarbons replacing the glucose).

Table 1. The oxygen uptake of *Proactinomyces opacus* in the presence of liquid paraffin, dodecane and tetradecane

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, incubated overnight. Next morning 0.2 ml. hydrocarbon and distilled water added to give a final volume of 2.5 ml.)

Addition	O ₂ Uptake in 120 min. (μl.)
None	36
Liquid paraffin	40
<i>n</i> -Dodecane	152
<i>n</i> -Tetradecane	92

respiration was 1.0. Saz (1949) found an identical value for one of his strains (H37Ra) using tetradecane. To account for the high value obtained he came to the conclusion that 'if the hydrocarbon is being oxidized, this would suggest that intracellular decarboxylation mechanisms are being stimulated as well'.

Reduction of methylene blue by extract from crushed cells

In order to obtain a sufficient weight of organism for this purpose, the organism was grown on the medium as previously given (see under Methods) but solidified with 2% agar. The poured plates were inoculated over the whole surface using a sterile glass spreader. The plates were incubated for 14 days at 23°, the surface growth was then harvested, washed once by centrifuging and then deposited in a tared tube by centrifuging. The cells were crushed by McIlwain's method (McIlwain, 1948), using Microid polishing alumina, Grade 3/50, slow cutting (Griffin and Tatlock Ltd.). After centrifuging, the supernatant was used for Thunberg experiments.

Table 4. *Reduction of methylene blue by the extract obtained from crushed cells of Proactinomyces opacus*

(The organisms (4.3 g. wet wt.) were crushed with 11 g. polishing alumina, taken up in 10 ml. phosphate buffer, pH 7.0, and centrifuged. The supernatant liquid was used in hooked Thunberg tubes, each of which contained 1 ml. of supernatant, 0.3 ml. 1/10 000 methylene blue and 0.2 ml. of substrate, etc., to give a total volume of 1.5 ml. Methylene blue and substrate were added from the 'hook' after evacuation and filling with O₂-free N₂.)

Addition	Time after which decoloration obtained (min.)
None	35
Dodecane	17
Hexadecane	17
Decane	40
Sodium lactate (0.2 ml. of 1%)	20

From Table 4 it will be seen that the rate of methylene-blue reduction is increased in the presence of dodecane and hexadecane. Decane was ineffective. It also had little effect on the oxygen uptake (Fig. 1). Sodium lactate has been included for purposes of comparison.

Metabolism of saturated aliphatic alcohols

Table 5 gives the results of an experiment with ethyl, butyl, amyl, isoamyl, isohexyl, heptyl, octyl, decyl, lauryl, cetyl and octadecyl alcohols. It is clearly seen that the long-chain alcohols (decyl, lauryl (dodecyl) and cetyl (hexadecyl)) have a

marked effect on the oxygen uptake, while butyl, amyl, isoamyl, isohexyl and heptyl alcohols are all toxic and no oxygen uptake is obtained in their presence. Saz (1949) found, too, that the oxygen uptake of the four strains of tubercle bacilli used by him were increased by long-chain (cetyl and stearyl (octadecyl)) alcohols. In the experiments

Table 5. *Oxygen uptake of Proactinomyces opacus in the presence of some saturated monohydric aliphatic alcohols*

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. 0.066M-phosphate buffer, pH 7.0, incubated overnight at 20°. Next morning 0.2 ml. of liquid alcohols + 0.2 ml. distilled water added from side arm. In case of solid alcohols 0.2 ml. of the melted alcohol was added directly to suspension. Distilled water added to give final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	15
Ethanol	104
Butanol	Nil
Amyl alcohol	Nil
isoAmyl alcohol	Nil
isoHexyl alcohol	Nil
Heptyl alcohol	Nil
Octyl alcohol	16
Decyl alcohol	76
Dodecyl alcohol	98
Hexadecyl alcohol	101
Octadecyl alcohol	47

with the immiscible liquid and solid hydrocarbons and alcohols some variation was encountered in the amount of increase in oxygen uptake observed for different experiments. We believe this is connected with the difficulties in introducing the exact amounts (particularly in the case of solid substances) into the main vessel and also to the different degrees of dispersion obtained. The substances in the absence of the cells did not take up oxygen.

Metabolism of saturated aliphatic fatty acids

When used in low concentrations (0.0012M) the longer chain acids give greater oxygen uptakes than acetate, propionate or butyrate (Fig. 2). This agrees with the findings of Randles (1950) for *Neisseria catarrhalis*. From Table 6 it will be seen that the very long-chain saturated fatty acids (laurate, myristate, palmitate, stearate and behenate) behaved in a similar way, although behenate is not so effective as the others.

In higher concentrations (1%, w/v) caprylate (octanoate), caprate (decanoate), undecanoate and laurate completely inhibited oxygen uptake. The toxic effect of high concentrations of caprylate, caprate, undecanoate and laurate may be connected with their detergent properties.

*Effect of fatty acids on oxygen consumption
in the presence of glucose*

The addition of the long-chain fatty acids (laurate and caprate) and glucose simultaneously to the washed suspension (Table 7) gave analogous results to those obtained for the hydrocarbons (see Table 3).

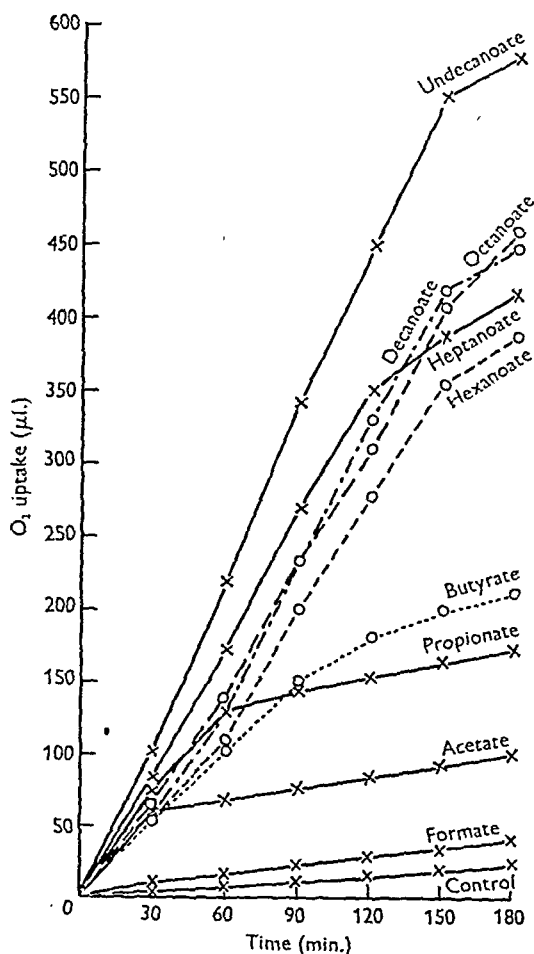


Fig. 2. The O_2 uptake of *Pro. opacus* in the presence of saturated aliphatic fatty acids. A washed suspension (1 ml.) of organism + 0.5 ml. phosphate buffer, pH 7.0, shaken gently overnight at 20°. Next morning substrates (0.5 ml. of 0.06M from side arm) and distilled water added to give a final concentration of 0.0012M-fatty acid as sodium salt on total volume of 2.5 ml.

DISCUSSION

Little work has been done on the metabolism of the partially acid-fast proactinomyces. (For details regarding acid-fastness in this group see Erikson (1949). The capacity of these organisms to grow on liquid paraffin and paraffin wax has been known for a long time (Jensen, 1931). From work reported in this paper it is clear that a wide range of hydrocarbons and closely related compounds increase the rate of oxygen uptake by washed suspensions of

Pro. opacus. Of the hydrocarbons utilized, the higher members, dodecane, tetradecane and hexadecane were superior to the other members tried (Fig. 1). In this respect *Pro. opacus* differs from the Gram-negative, non acid-fast pseudomonas species as used by Johnson *et al.* (1942), who found that octane was the most actively used substance. Saz (1949), working with pathogenic strains of mycobacteria, obtained similar results to ourselves. He suggested two possibilities to account for the increased oxygen uptake: (a) that the hydrocarbons are used as energy source by the organism, and (b) that the hydrocarbons alter the respiration rate by changing the total free surface of the cells.

Table 6. The oxygen uptake of *Proactinomyces opacus* in the presence of long-chain fatty acids

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning 0.5 ml. of 0.006M-sodium salt of acid tipped from side arm. Distilled water added to give final concentration of 0.0012M and final volume of 2.5 ml.)

Addition	O_2 uptake in 120 min. (μ l.)
None	17
Laurate (dodecanoate)	204
Myristate (tetradecanoate)	275
Palmitate	214
Stearate	193
Behenate (docosoate)	37

Table 7. The effect of fatty acids on the oxygen consumption of *Proactinomyces opacus* in the presence of glucose

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning substrates and distilled water added to give final volume of 2.5 ml.)

Addition	O_2 uptake in 120 min. (μ l.)
None	47
Glucose (0.4%, w/v)	195
Sodium dodecanoate (0.0012M)	225
Glucose (0.4%, w/v) + sodium dodecanoate (0.0012M)	366
Sodium decanoate (0.0012M)	134
Sodium decanoate (0.0012M) + glucose (0.4%, w/v)	242

In the case of *Pro. opacus*, good growth is obtained using dodecane and tetradecane as sole carbon source in the medium. Saz (1949), on the other hand, only obtained stimulation of growth of one of his strains. The other strains tested were apparently unable to utilize the hydrocarbons as energy source.

As to the question of whether the oxygen uptake observed represents a true oxidation of the substrate added, our growth experiments would

support the view that the hydrocarbons are being actively metabolized. This is further borne out by the results obtained on the partially oxidized derivatives, such as long-chain monohydric alcohols and long-chain monocarboxylic acids. Methylene-blue reduction, moreover, points to the presence of a dehydrogenase enzyme present in the cells capable of oxidizing the long-chain hydrocarbons.

It seems that this organism offers very interesting possibilities for further studies on metabolism.

SUMMARY

1. The oxygen uptake of washed suspensions of *Proactinomyces opacus* is increased in the presence of *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane and paraffin wax.

2. Decyl, lauryl (dodecyl) and octadecyl alcohols give increased oxygen uptake, but amyl, isoamyl, isohexyl, and heptyl alcohols are toxic.

3. The long-chain fatty acids (C_7 – C_{16}) are all actively metabolized at very low concentrations (0.0012M). At 1%, octanoate, decanoate, undecanoate and laurate (dodecanoate) are toxic.

4. The rate of methylene-blue reduction by the supernatant liquid obtained from the crushed cells of *Proactinomyces opacus* is increased in the presence of dodecane and hexadecane.

We are grateful to Mrs D. Oxford for suggesting this problem and to her and to Dr G. K. Fraser for their continued interest in this work. The work was carried out in the Botany Department, and we are grateful to Prof. J. R. Matthews for the facilities provided. We also wish to thank Messrs Hess Products Ltd., Littleborough, for gifts of fatty acids and to others for gifts of hydrocarbons of high purity.

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Reduction of Dehydroascorbic Acid by Bacteria

2. ROLE OF CYTOCHROME IN HYDROGEN TRANSPORT

By B. P. EDDY, M. INGRAM AND L. W. MAPSON

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

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In a previous paper (Eddy, 1952) it was shown that the reduction of dehydroascorbic acid (DHA) to ascorbic acid (AA) by *Escherichia coli* can be effected with hydrogen supplied by various hydrogen donor/dehydrogenase systems in intact cells, but that corresponding cell-free systems are ineffective. It was therefore suggested that some additional factor, possibly a hydrogen carrier, is necessary.

Our attention was first directed to cytochrome because it was reported that some fifteen strains of streptococci were unable to reduce DHA (Gunsalus & Hand, 1941) and we found the same with species of *Lactobacillus* and *Clostridium*. These bacteria can produce reducing conditions of sufficiently high intensity for the reduction of DHA to be thermodynamically possible, but possess no cytochrome (Fujita & Kodama, 1934; Frei, Riedmüller &

Almasy, 1934). When a connexion between DHA reduction and cytochrome was in fact found in *Esch. coli*, comparison of the two properties was naturally extended over a range of bacterial types.

The present paper falls, therefore, into two parts, the first describing attempts to relate DHA reduction to the hydrogen carrier and the second being a survey of the types of bacteria able to effect the reduction.

METHODS

For spectroscopic work the organisms were grown in bulk on a solid medium containing 1% Protex, 1% peptone, 1% gelatin, 0.5% Yeastrel, 0.5% glucose, 1% $CaCO_3$ and 3% agar. After harvesting, the cells were washed twice with Ringer's solution (quarter strength) and suspended at the required concentration either in this solution or in phosphate buffer at pH 6.2.

Organisms to be tested for DHA reduction were grown in nutrient broth + 0.5% glucose in 1 oz. screw-capped bottles in which they were centrifuged, washed once with Ringer's solution and finally suspended in buffer. Their ability to reduce DHA was tested as described previously (Eddy, 1952) using 0.02M-glucose as hydrogen donor.

For viewing cytochrome spectra a Zeiss microspectroscope was used. Investigations at liquid air temperature were carried out as described by Keilin & Hartree (1949). For estimating changes in the intensities of cytochrome bands the microspectroscope was fixed vertically above one limb of a Duboscq colorimeter from which the prisms and eyepiece had been removed. A standard suspension of *Esch. coli* (1.5×10^{11} cells/ml.), with added dithionite, was placed in the colorimeter cup and the vernier scale set at 10 mm. The experimental suspension of equal density but without dithionite was placed in a cell of optical depth 10 mm., flooded with liquid paraffin and illuminated horizontally so that both spectra could be viewed simultaneously. The intensities of the spectra were then matched by altering the intensities of their respective light sources. After the addition of DHA the intensities of the spectra and of the cytochrome b_1 bands were rematched, altering the comparison spectrum by varying the intensity of the light source and the depth of suspension interposed.

RESULTS

A. Connexion between cytochrome and ability to reduce DHA in *Escherichia coli*

It seemed that, if cytochrome were needed to reduce DHA, the failure of enzyme preparations to effect the reduction might be due to the absence of cytochrome. Non-reducing cell-free dehydrogenase preparations of *Esch. coli* prepared by tryptic digestion and acetone treatment as described in a previous paper (Eddy, 1952), or mechanically in a Mickle tissue disintegrator, were examined spectroscopically after the addition of sodium dithionite. It was found that in all cases the cytochrome bands were either absent or were so weakened as to be barely visible; this supported the idea that reduction might be connected with cytochrome.

Unfortunately we have not been able to obtain cell-free preparations of the cytochrome from *Esch. coli*, with which to try to restore the reducing capacity of cell-free dehydrogenase preparations, and so we have had to use spectroscopic methods to detect any reaction between DHA and the cytochrome. The experiments have consisted essentially in generating the reduced cytochrome spectrum (b_1 band) by incubating a suspension of *Esch. coli* anaerobically, and then adding DHA (also anaerobically because the band disappears completely on aeration), and seeking to observe a weakening of the absorption band due to oxidation of the cytochrome by the DHA.

The first observations were made visually. They indicated that the intensity of the b_1 band was decreased on adding DHA, but only slightly. Because it was difficult visually to be certain of the

oxidation of the band, when it was so incomplete, attempts were made to confirm the observations photographically, using the following technique.

A suspension of *Esch. coli* containing approximately 1.5×10^{11} cells/ml. was prepared in phosphate buffer at pH 6.2. 2.5 ml. of this suspension were transferred to a glass cell of optical depth 5 mm. and the surface was flooded with liquid paraffin to maintain anaerobic conditions in the system. 0.1 ml. 0.0625M-sodium formate was added (at the final concentration the dehydrogenase activity

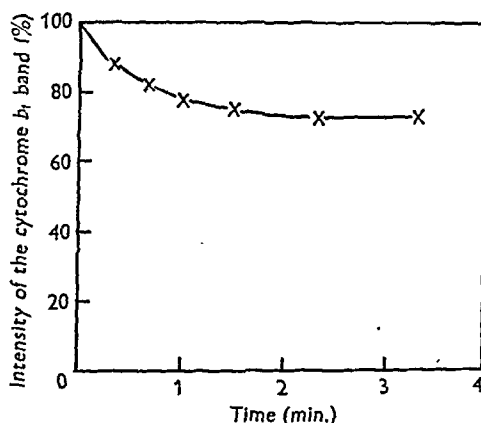


Fig. 1. Effect of dehydroascorbic acid on the fully reduced cytochrome b_1 band in *Esch. coli*. 1 mg. DHA was added to 2.5 ml. suspension containing 1.5×10^{11} cells/ml.

would be about half maximal), the cytochrome allowed to become fully reduced and the spectrum photographed. Urethane (0.1 ml. 50%, w/v), DHA (0.1 ml. 1% (w/v); to give maximal rate of reduction with respect to DHA concentration) and finally sodium dithionite, were added in turn and the spectrum photographed after each addition. Both the urethane and DHA solutions were previously de-aerated by evacuation and held under liquid paraffin until required. A definite weakening of the cytochrome b_1 band was recorded photographically after the addition of DHA in such experiments.

Attempts were then made to measure the degree of oxidation of the cytochrome b_1 band, by comparing it in the microspectroscope with that of a standard suspension of *Esch. coli* in which the cytochrome had been completely reduced with dithionite. Using a suspension in which the bands had become fully reduced without added hydrogen donor, and to which urethane had then been added, it was estimated that the addition of DHA resulted in approximately 30% diminution in the intensity of the cytochrome b_1 band in 3 min. (Fig. 1), indicating 30% oxidation of the reduced cytochrome. When the urethane was omitted, and sufficient glucose included to reduce all the added DHA, the cytochrome (b_1 band) changed from 70 to

90% reduced in 2.5 hr. and could then be re-oxidized to the same extent as previously by the addition of more DHA.

These observations appeared to reveal a connexion between the cytochrome of *Esch. coli* and its ability to reduce DHA. It was soon evident, however, that some organisms possessing cytochrome were unable to effect this reduction: e.g. baker's yeast had been reported to be ineffective by Gunsalus & Hand (1941) and we confirmed this observation several times. It therefore seemed necessary to compare the ability to reduce DHA with the cytochrome complement in a representative range of bacterial types.

B. A survey of DHA-reducing ability and cytochrome complement in different genera

Some information was already available. The cytochrome absorption spectra for a variety of bacteria had been listed by Fujita & Kodama (1934) and by Frei *et al.* (1934). These lists were a guide in choosing types for test, though we always checked the quoted complement against that actually observed in the strains we used. Some information had also been published about the types of organisms able to reduce DHA, and as Stewart & Sharp (1945) had reported that various staphylococci and coliform bacteria are best able to bring about this reduction, we began by testing related organisms.

Representative species of *Eberthella*, *Erwinia*, *Escherichia*, *Proteus*, *Salmonella* and *Serratia* were found to reduce DHA. Gunsalus & Hand (1941) reported that several strains of *Aerobacter cloacae* could also effect the reduction, though a British strain and two strains of *A. aerogenes* tested by us were found to be ineffective. Strains of three species of *Staphylococcus* were also found to be able to reduce DHA. The nomenclature in this paper follows *Bergey's Manual of Determinative Bacteriology*, 5th ed. (1939).

Species from most of the above genera had been examined by Fujita & Kodama (1934) and found to exhibit the cytochrome b_1 band in place of the b and c bands of other types. Hence a series of types characterized by other cytochrome complements was next examined—species of *Clostridium* (1), *Lactobacillus* (1), *Acetobacter* (2), *Pseudomonas* (1), *Bacillus* (8), *Micrococcus* (7) and *Sarcina* (1)—and all were found to be unable to reduce DHA.

These observations suggested a correlation between ability to reduce DHA and the possession of cytochrome b_1 , and our belief in this connexion was strengthened on careful examination of several apparently irregular cases:

(1) It had been reported (Tkachenko, 1936) that DHA was reduced by *Lactobacillus acidophilus*. As species of this genus possess no cytochrome, we

carefully examined three strains of this species, and found no evidence of reducing power. Reference to the technique employed by Tkachenko (1936) has led us to regard his evidence as inconclusive.

(2) On examination of a strain designated *Staphylococcus citreus* which failed to reduce DHA, it was found to possess a spectrum with well defined cytochrome b and c bands, and further systematic examination revealed that it was, in fact, *M. conglomeratus*. It thus fell into line with the other *Micrococcus* species examined, namely *M. aurantiacus*, *M. candicans*, *M. flavus*, *M. freudenreichii*, *M. luteus*, *M. lyso-deikticus* and *M. ureae*.

(3) Among the Gram-negative bacilli related to the coliforms and included in the family Enterobacteriaceae according to *Bergey's Manual*, 5th ed. (1939), Fujita & Kodama (1934) described one species (*Bacillus prodigiosus* syn. *Serratia marcescens*) as possessing cytochromes b and c . As we found species of *Serratia* able to reduce DHA, we examined their spectra and found that they possessed only a single band, and that the band remained single even on cooling in liquid air, a treatment which sharpens the bands and makes it possible to distinguish readily cytochromes b and c (Keilin & Hartree, 1949).

(4) The situation in the genus *Bacillus* was particularly interesting. Our examination of strains of eight species (*B. cereus*, *B. licheniformis*, *B. megatherium*, *B. mesentericus*, *B. mycoides*, *B. polymyxa*, *B. pumilus* and *B. subtilis*) showed that none could reduce DHA though, on the other hand, Keilin (1934) had earlier reported that *B. megatherium* possessed a cytochrome b_1 band similar to that in *Esch. coli* and similar reports had been made in respect of *B. subtilis* (Frei *et al.* 1934). Further, Chaix & Roncoli (1951) have recently observed that young cultures of several *Bacillus* species exhibit a single broad absorption band at 550–560 $m\mu$, which they provisionally identify as that of cytochrome b_1 . Examination of the spectra of our *Bacillus* strains (in the state in which we tested them for DHA reduction) showed that they mostly possessed single cytochrome bands similar to those described by Chaix & Roncoli. At first, these observations weakened our belief in the connexion between DHA reduction and cytochrome b_1 . However, careful examination showed that the cytochrome bands in these *Bacillus* strains were broader than the typical b_1 bands of staphylococci and the Enterobacteriaceae. On heating a suspension of *B. pumilus* to 80° for 20 min. in the presence of dithionite, the long-wavelength side of the band disappeared, leaving a narrow band in the position of the typical c band. Again, examination of the ' b_1 ' band in *B. cereus* at the temperature of liquid air showed a band centred at 552 $m\mu$. with faint shading on either side (possibly indicative of

two additional bands analogous to the spectrum found in old cultures of *B. subtilis* which contains cytochromes *b* and *c*), whereas under these conditions the coliforms show a single band from 553 to 560 m μ . with a barely perceptible maximum at 557 m μ . These observations lead us to conclude that the single band in *Bacillus* species is probably not identical with the classical *b*₁ band of the coliform organisms, which conclusion agrees with the inability of the *Bacillus* strains to reduce DHA.

It seems, therefore, that ability to reduce DHA is confined to some strains within the genus *Staphylococcus* and the family Enterobacteriaceae, which contain typical cytochrome *b*₁.

Variation in DHA-reducing capacity among strains. It has become certain, however, that a considerable proportion of organisms apparently containing a perfectly normal cytochrome *b*₁ are unable to reduce DHA. On further investigation of the two groups of reducing organisms mentioned above, it was found that some members of each were unable to reduce DHA. Six strains each of *Staph. albus* and *Staph. aureus* were provided by Dr R. M. Fry (Public Health Laboratory, Cambridge) and two strains of *Staph. citreus* (7415, 7990) were obtained from the National Collection of Type Cultures (N.C.T.C.). Of these, one strain of *Staph. albus* failed to reduce DHA. Table 1 gives the

(1945) stated that, of the strains of coliform organisms they tested, about half did not reduce DHA.

With a strain of *Esch. coli* (N.C.T.C. 7277) which is unable to reduce DHA, experiments similar to those in section A did not reveal any alteration in the intensity of the cytochrome band on addition of DHA. Attempts to make similar observations with strains of *Staphylococcus* were indecisive because the cytochrome bands were too weak.

DISCUSSION

The results in the first half of the preceding section, suggesting on spectroscopic evidence that cytochrome *b*₁ is oxidized by DHA, support, and are supported by, those in the second half, which indicate that a grouping of bacteria according to their complement of cytochromes will delimit a group of genera within which the ability to reduce DHA is confined. Taken together, the observations show that DHA can be reduced by hydrogen transported by cytochrome *b*₁ but not by other cytochromes.

These relations, be it noted, are those which obtain when dehydrogenases of the anaerobic type effect the reduction of DHA. With other hydrogen-donating systems different relations may hold: for example, glycine can act as a hydrogen donor for

Table 1. *Ability to reduce dehydroascorbic acid among members of the family Enterobacteriaceae*

(Numbers quoted are those of N.C.T.C. strains; other strains were supplied by Dr W. J. Dowson, Botany School, Cambridge.)

Species	Reducing strains	Non-reducing strains
<i>Aerobacter aerogenes</i>	—	(2 unknown)
<i>A. cloacae</i>	(U.S. strains)	(1 unknown)
<i>Eberthella belfastiensis</i> (<i>Bact. coli anaerogenes</i>)	4450	4174
<i>Erwinia aroidae</i>	(1 unknown)	—
<i>Erwinia carotovora</i>	(4 unknown)	—
<i>Escherichia coli</i> (<i>Bact. coli</i> Type I)	8196, (1 unknown)	8179, 8333
<i>Esch. coli</i> (<i>Bact. coli</i> Type II)	8164	—
<i>Esch. coli</i> (irregular)	—	7275, 7277
<i>Esch. freundii</i> (<i>Bact. coli</i> Inter. I)	—	8165, 8166
<i>Esch. freundii</i> (<i>Bact. coli</i> Inter. II)	6072	6071
<i>Klebsiella ozaenae</i>	—	5053
<i>K. pneumoniae</i>	—	2794, (1 unknown)
<i>K. pneumoniae A</i>	—	5054
<i>K. pneumoniae B</i>	—	5055
<i>K. pneumoniae C</i>	—	5056
<i>K. rhinoscleromatis</i>	—	5047
<i>Proteus vulgaris</i>	(1 unknown)	—
<i>Salmonella pullorum</i>	(1 unknown)	—
<i>Serratia marcescens</i>	(1 unknown)	(1 unknown)
<i>Serratia kielensis</i>	4619	—

occurrence of DHA-reducing ability in the strains of species from the Enterobacteriaceae which we have examined; about half are inactive. Similar results had in fact been recorded previously. Gunsalus & Hand (1941) and Stewart & Sharp

DHA reduction (Eddy, 1952, Table 1), but the dehydrogenase probably involved is an autoxidizable flavoprotein. Such a system might not be expected to show any relation to cytochrome, but the fact that cytochrome is not an essential inter-

mediate does not exclude the possibility that the dehydrogenase is capable of reducing it.

It is not known why the cytochrome b_1 band is not fully removed by DHA. It may be either that the residual level of dehydrogenase activity in our experiments was sufficient to keep the cytochrome 70% reduced or, alternatively, that the oxidation-reduction potential of the AA/DHA system, as constituted in this case, was the same as that of 70% reduced cytochrome b_1 .

The inability of many organisms containing cytochrome b_1 to effect the reduction raises a further problem. As there is no evidence that this is due either to their cytochromes or cytochrome-reducing mechanisms, we suppose it to be due to lack of a mechanism linking oxidation of the cytochrome with reduction of DHA. In the previous paper (Eddy, 1952) theoretical considerations were given in favour of an enzyme activating DHA so that it can be reduced. The failure of some coliform bacteria to reduce DHA lends weight to these suggestions, and preliminary experiments have shown that the oxidation of leuco-Nile blue by DHA varies both in rate and magnitude, according to whether DHA-reducing or DHA-non-reducing strains of bacteria are present.

SUMMARY

1. In cell-free dehydrogenase preparations from *Escherichia coli*, which are unable to reduce dehydroascorbic acid (DHA), the cytochromes are either absent or greatly decreased in amount.

2. When DHA is added anaerobically to a suspension of *Escherichia coli* the intensity of the fully reduced cytochrome b_1 band becomes diminished by approximately 30%.

3. Amongst organisms examined so far, ability to reduce DHA is confined to members of the genus *Staphylococcus* and the family Enterobacteriaceae, these being the groups in which the b and c cytochrome bands are replaced by the b_1 band.

4. Approximately half the members of the Enterobacteriaceae tested failed to reduce DHA, and it is suggested that a DHA-activating enzyme may be necessary in addition to cytochrome b_1 .

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Studies in Immunochemistry

11. THE ACTION OF DILUTE ALKALI ON THE N-ACETYLHEXOSAMINES AND THE SPECIFIC BLOOD-GROUP MUCOIDS

By D. AMINOFF, W. T. J. MORGAN AND WINIFRED M. WATKINS
The Lister Institute of Preventive Medicine, London, S.W. 1

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The increasing use within recent years of the colorimetric method introduced by Morgan & Elson (1934) for the detection and quantitative determination of the N-acetylhexosamines calls for a more detailed study of the conditions necessary to obtain reliable and reproducible results and demands a more thorough understanding of the structural changes involved before the results can

be applied to complex structures containing N-acetylhexosamine components, such as the blood-group mucoids.

Ehrlich (1901) observed that mucins and mucoid substances give rise to an intense purple colour after heating with dilute alkali for a short time and the addition of an acid solution of *p*-dimethylaminobenzaldehyde. If the alkali treatment is

omitted no colour formation takes place. At about the same time Müller (1901) found that penta-acetylglucosamine after heating with dilute potassium hydroxide gave a similar colour on the addition of Ehrlich's *p*-dimethylaminobenzaldehyde reagent, and Zuckerkandl & Messiner-Klebermass (1931) subsequently suggested that a pyrroline derivative was formed from *N*-acetylglucosamine by the action of alkali, and that this substance reacted with Ehrlich's reagent to yield a coloured complex. Morgan & Elson (1934) and Morgan (1938) considered that the formation of a pyrrol derivative was difficult to reconcile with the fact that *N*-trimethylacetyl-, *N*-*o*-bromobenzoyl- and *N*-benzoyl-glucosamines gave a similar colour after treatment with dilute alkali and Ehrlich's reagent and it was suggested that *N*-acetylglucosamine formed an oxazole or oxazoline derivative under these conditions. Some oxazole preparations, made by condensing amides with α -halogenoketones (Blumlein, 1884; Lewy, 1887) gave, without previous alkali treatment, a strong colour with the *p*-dimethylaminobenzaldehyde reagent, and the production of an immediate colour by these substances seemed to support the suggestion that *N*-acetylglucosamine was converted to a heterocyclic structure of this kind. It has been known for some time, however, that the positive colour reaction given by the oxazole preparations is due to the presence of an impurity of unknown nature in the materials examined and that if the oxazole is purified by precipitation as a complex with mercuric chloride (Cornforth & Cornforth, 1947), it fails to give a positive colour reaction with *p*-dimethylaminobenzaldehyde.

A number of authentic oxazoline derivatives were found by Morgan (unpublished observations) to yield no colour with the *p*-dimethylaminobenzaldehyde reagent, but nevertheless White (1940), on the basis of the results of methylation experiments, concluded that the formation of a glucoxazoline (2-methyl-4:5-glucopyrano- Δ^2 -oxazoline) occurred when *N*-acetylglucosamine was treated with alkali. Morgan (1938) pointed out that two molecules of *N*-acetylglucosamine might condense together and form a diglucopyrazine structure similar to that proposed earlier by Stolte (1908), who claimed to have obtained ditetrahydroxybutylpyrazine from glucosamine.

In an attempt to understand more fully the structural changes which occur when the *N*-acetylhexosamines are treated with dilute alkali under controlled conditions, such as those employed for their colorimetric determination, a study has been made of the ultraviolet absorption spectra given by these substances after treatment with dilute sodium carbonate or with different buffer systems at known pH values, and of the light absorption of

the systems after the development of the purple colour following the addition of the *p*-dimethylaminobenzaldehyde reagent. A number of purified human blood-group substances which are mucoids and are known to behave with alkali and Ehrlich's reagent as do the *N*-acetylhexosamines, have been examined in the same manner. The isolation and identification of the substance which arises from the simple *N*-acetylhexosamines on treatment with alkali and which is responsible for giving the colour with Ehrlich's reagent is being undertaken in collaboration with Dr J. W. Cornforth.

EXPERIMENTAL

Materials and methods

N-Acetylglucosamine. A standard solution of *N*-acetylglucosamine (1%) was kept at 2° and from this, suitable dilutions were made immediately before use.

N-Acetylchondrosamine. The substance was prepared by the acetylation of chondrosamine hydrochloride with acetic anhydride in the presence of silver acetate. (Found: C, 42.9; H, 6.7; N, 6.3; $C_8H_{15}O_6N$ requires C, 43.4; H, 6.8; N, 6.3%) $[\alpha]_{5461}^{20} + 131^\circ \rightarrow 98^\circ$ (c, 0.5 in water); m.p. 162–164° (uncorr.). The material was found to be chromatographically homogeneous, a single component being observed when a solution of the substance was run in collidine, butanol, butanol-acetic acid, butanol-ethanol or in phenol according to the standard techniques used in paper chromatography. The R_F value of the material in these solvents is very similar to that of *N*-acetylglucosamine run simultaneously. After hydrolysis with 0.5*N*-HCl for 16 hr. at 100°, the material gave the full hexosamine colour corresponding to an equivalent weight of glucosamine hydrochloride and chromatographic analysis of the hydrolysate revealed the presence of one component only, which had all the properties of chondrosamine hydrochloride. A 1% solution of *N*-acetylchondrosamine was kept at 2° and the appropriate dilutions made immediately before the experiment.

p-Dimethylaminobenzaldehyde reagent (DMAB). A.R. grade *p*-dimethylaminobenzaldehyde was further purified by the method of Adams & Coleman (1944) and finally fractionated by the addition of water to an ethanol solution of the material. The crystals separating from solution within the range 50–75% water are almost colourless and are suitable for use. The reagent was prepared by dissolving 2 g. of the material in 100 ml. of A.R. glacial acetic acid which contains 2.5% (v/v) A.R. 10*N*-HCl. The solution, which possessed a very pale greenish yellow colour, was stored at 0° in small glass-stoppered bottles. A fresh bottle was opened for each experiment. The use of material which has been repeatedly thawed and frozen or allowed to stand at room temperature should be avoided.

Reducing sugars. Determined according to Somogyi (1937).

Fucose. Determined by the method of Dische & Shettles (1948).

Glucosamine and chondrosamine. Determined as described by Elson & Morgan (1933) using hexosamine concentrations of 10–40 μ g. as standards, a photoelectric colorimeter and a green filter (max. transmission 550 $m\mu$), or a Hilger Uvispek spectrophotometer.

Buffer solutions. As described by Vogel (1939).

A substance. The material was obtained from a pseudomucinous cyst fluid (no. 122) according to the isolation procedures described by Aminoff, Morgan & Watkins (1950). $[\alpha]_{5461} + 11^\circ$. N, 5.5% (Kjeldahl). Total fucose, 18.3%. Reducing power, expressed as glucose after acid hydrolysis, 54%. Hexosamine, as base, 32%.

B substance. The material was prepared from an ovarian cyst fluid by Mr R. A. Gibbons. The substance showed general chemical properties similar to those recorded for group A substance. N, 5.8%. Total fucose, 18%. Reducing power, expressed as glucose after hydrolysis, 50%. Hexosamine, as base, 20%.

H substance. Prepared according to Morgan & Waddell (1945) and Annison & Morgan (unpublished) from cyst fluids obtained from 'secretors' belonging to group O. The material was found to be similar in general chemical properties to the groups A and B substances. N, 5.3%. Total fucose, 14%. Reducing power, expressed as glucose after hydrolysis, 54%. Hexosamine, as base, 31%. $[\alpha]_{5461} - 30^\circ$ (c, 0.5 in water).

'Lewis' Le^a substance. Prepared as described by Annison & Morgan (1951) from a cyst fluid obtained from a 'Lewis-positive' patient. The substance, $[\alpha]_{5461} - 41^\circ$, contained N, 5.4%, total fucose, 13% and gave, after hydrolysis with 0.5N-HCl at 100° , 57% reducing sugars (as glucose) and 32% hexosamine (as base).

Optimal conditions for the colorimetric determination of the N-acetylhexosamines

A re-examination of the general procedure used by Morgan & Elson (1934) for the estimation of the *N*-acetylhexosamines was first undertaken.

Period of heating with alkali. *N*-Acetylglucosamine (20 μ g.) was measured into each of several test tubes (20 \times 1 cm.) graduated at 10 ml. capacity which were carefully selected to possess walls of equal thickness. The sides of the tubes were washed down with water to yield a total volume of 1 ml., and 0.1 ml. of 0.5N- Na_2CO_3 was added and the contents thoroughly mixed. Each tube was closed by a glass ampoule, the sealed neck of which was inserted into the tube. The ampoules contained 2-3 ml. of water and acted as condensers which prevented loss by evaporation during the short heating periods studied. The tubes were heated up to the level of the liquid contents in a vigorously boiling water bath, and at pre-arranged times up to a maximum period of 15 min., pairs of tubes were withdrawn from the bath and immediately cooled in water at 0° and kept there until all tubes had been heated and cooled. Glacial acetic acid (A.R.) was then run into each tube from a separating funnel to yield a total volume of about 7 ml. and the DMAB reagent (1 ml.) was added. The volume of liquid in each tube was made up to 10 ml. by the addition of glacial acetic acid, was thoroughly mixed and the whole series of tubes allowed to stand at 20° in the dark. A tube containing all the reagents other than the *N*-acetylhexosamine was included to serve as a reagent blank in each experiment. The colour intensity of each solution reached a maximum value after standing for 1.5 hr. at 20° . For routine purposes the intensity of the colour developed is conveniently measured in a simple photoelectric colorimeter using a green filter (max. transmission 550 $\text{m}\mu$.) and optical cells 2 cm. deep. In our experience it is advisable to mix the

contents of each tube again a few minutes before they are examined in the colorimeter or spectrophotometer.

The relationship between the time of heating with carbonate solution and the intensity of the colour subsequently produced with a fixed amount of DMAB reagent and expressed directly in terms of the galvanometer readings is given in Fig. 1. Under the conditions described the maximum colour was given by a solution which had been heated for 4 min. and it is evident that after this time the structure which gives rise to the coloured complex with the DMAB reagent undergoes fairly rapid destruction, and it is therefore important that the period of heating with alkali should be accurately controlled. The length of the heating period which gives the optimal colour production on the addition of the DMAB reagent varies somewhat according to the thickness and shape of the glass tubes selected, the heating conditions etc., and must be determined for each outfit.

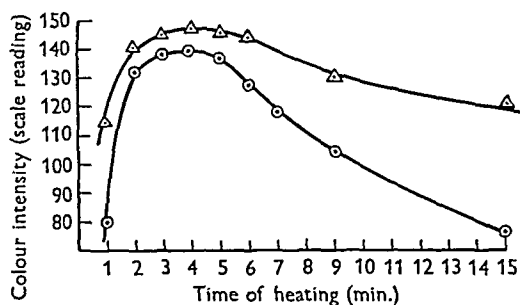


Fig. 1. The influence of the time of heating with Na_2CO_3 on the intensity of the colour produced on the addition of DMAB reagent. \bigcirc — \bigcirc , *N*-acetylglucosamine; \triangle — \triangle , *N*-acetylchondrosamine.

A similar curve (Fig. 1) was obtained for *N*-acetylchondrosamine, but it was found that nearly five times as much of this amino sugar was required to give a solution of equal colour intensity. Several preparations of *N*-acetylchondrosamine gave similar results. The *N*-acetylchondrosamine chromogen which gives rise to the coloured DMAB complex is apparently less rapidly destroyed than is that derived from *N*-acetylglucosamine. The 4 min. heating period was optimal for quantities from 10 to 50 μ g. of the two *N*-acetylhexosamines treated as described above.

Concentration of alkali. The influence of the concentration of Na_2CO_3 and of the period of heating on the amount of colour subsequently developed by *N*-acetylglucosamine after the addition of the DMAB reagent was next examined. Amounts (20 μ g.) of *N*-acetylglucosamine were placed in a series of tubes with a solution of Na_2CO_3 of known strength and the total volume of the solution made up to 1.1 ml. with water. The tubes were then heated in a boiling-water bath for predetermined periods, removed in pairs, cooled and treated with glacial acetic acid and DMAB reagent. Duplicate tubes containing 10, 20 and 30 μ g. of *N*-acetylglucosamine were likewise heated for 4 min. to act as standards and the maximum colour intensity reached by each pair of tubes, expressed in terms of that given by the *N*-acetylglucosamine standard, was plotted against the time of heating with alkali. The results obtained when three series of tubes containing 0.1, 0.4 and 0.8 ml. of 0.125N- Na_2CO_3 respectively were examined are given in Fig. 2. *N*-Acetyl-

chondrosamine (100 $\mu\text{g.}$) was treated in the same manner and the results, expressed in terms of the colour given by *N*-acetylchondrosamine heated for 4 min., are given in Fig. 3. It appears that under these conditions *N*-acetylchondrosamine requires a slightly shorter period of heating with alkali to yield subsequently its maximum colour than does *N*-acetylglucosamine.

same pH (10.8), and with the same buffers at different pH values, on the maximum amount of colour finally obtained was investigated, using 0.2 ml. of buffer solution in place of the 0.1 ml. of 0.5*N*- Na_2CO_3 used in the 'standard' test. Where the heating period necessary to yield the maximum colour exceeded 30 min. the heating was carried out in sealed glass ampoules which contained a total volume of

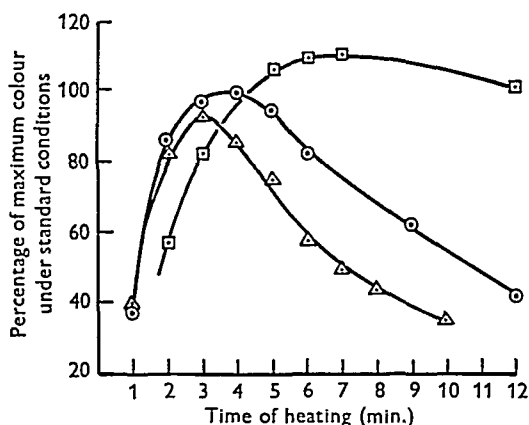


Fig. 2. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylglucosamine on the addition of DMAB reagent. \square — \square , 0.1 ml. 0.125*N*- Na_2CO_3 ; \circ — \circ , 0.4 ml. 0.125*N*- Na_2CO_3 ; \triangle — \triangle , 0.8 ml. 0.125*N*- Na_2CO_3 .

Conditions for the standard test. The results of these experiments allowed a set of conditions, the so-called 'standard' test conditions, for the estimation of the *N*-acetylhexosamines to be established. Thus, to a neutral solution of the test material contained in a total volume of 1.0 ml., 0.10 ml. of 0.5*N*- Na_2CO_3 is added and the solution is heated for 4 min. in a vigorously boiling water bath, cooled and treated as described above. Standard solutions each containing 10, 20 and 30 $\mu\text{g.}$ of *N*-acetylglucosamine or 50, 100 and 150 $\mu\text{g.}$ *N*-acetylchondrosamine, according to the aminohexose being examined, are included in each determination made. Under the conditions described the pH of the reactants during the heating period is about 10.8.

Influence of different buffer systems and pH values on the rate and intensity of colour development. The effect of heating *N*-acetylglucosamine with different buffer systems at the

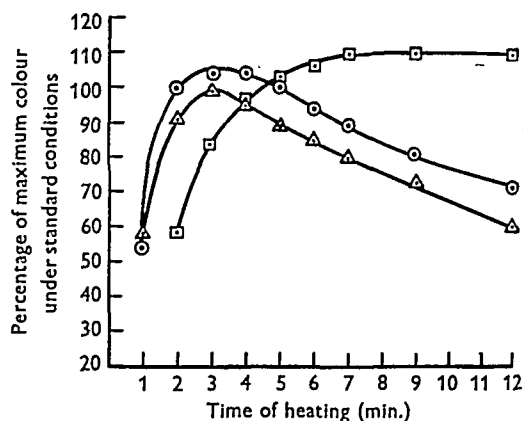


Fig. 3. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylchondrosamine on the addition of DMAB reagent. \square — \square , 0.1 ml. 0.125*N*- Na_2CO_3 ; \circ — \circ , 0.4 ml. 0.125*N*- Na_2CO_3 ; \triangle — \triangle , 0.8 ml. 0.125*N*- Na_2CO_3 .

approximately 1.3 ml. and, subsequently, amounts (1.1 ml.) of the cooled reaction mixture which contained exactly 20 $\mu\text{g.}$ of *N*-acetylglucosamine were pipetted into calibrated test tubes, glacial acetic acid and the DMAB reagent added, and the colours allowed to develop in the usual way. The results obtained are given in Table 1. It was observed many years ago that the use of borate buffer at about pH 10.8 in place of Na_2CO_3 at the same pH gave rise to considerably more colour, and the results set out in Table 1 confirm this early observation. The amount of colour obtained on addition of DMAB reagent to *N*-acetylglucosamine heated with glycine buffer at pH 10.8 was almost identical with that obtained with Na_2CO_3 .

The experiments were repeated using *N*-acetylchondrosamine, and the results (Table 1) revealed at once that the two amino sugars behaved qualitatively in a similar manner

Table 1. Amount of colour given by the *N*-acetylhexosamines after treatment at 100° with different buffer solutions and the addition of DMAB reagent

		<i>N</i> -Acetylglucosamine		<i>N</i> -Acetylchondrosamine	
		Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine under standard conditions	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine under standard conditions
Alkaline system	pH				
'Standard' procedure	10.8	4	100	3-4	23
Potassium borate + KOH	9.0	45	152	50	40
	10.0	12	155	15	35
	11.0	7	156	10	30
Glycine buffer	10.0	110	97	90	25
	10.9	40	100	40	25
	12.2	2	77	1-2	12

with the different buffer systems but that in each instance *N*-acetylchondrosamine gave rise to a maximum amount of colour equivalent to about a fifth of that given by an equal weight of *N*-acetylglucosamine.

Concentration of *p*-dimethylaminobenzaldehyde. The influence of the concentration of *p*-dimethylaminobenzaldehyde in the DMAB reagent on the intensity of the colour formed was investigated by the standard procedure. The DMAB reagents used in the test series contained 1, 2 and 4% (w/v) *p*-dimethylaminobenzaldehyde. The results indicated that increasing concentrations of *p*-dimethylaminobenzaldehyde in the Ehrlich's reagent give rise to a small increase in colour for a constant amount of the *N*-acetylhexosamine. The advantage gained by the development of an increased intensity of colour when using a more concentrated solution of DMAB, however, is offset to some extent by a corresponding increase in the correction necessary to compensate for the enhanced yellowish green coloration given by the reagent's 'blank'.

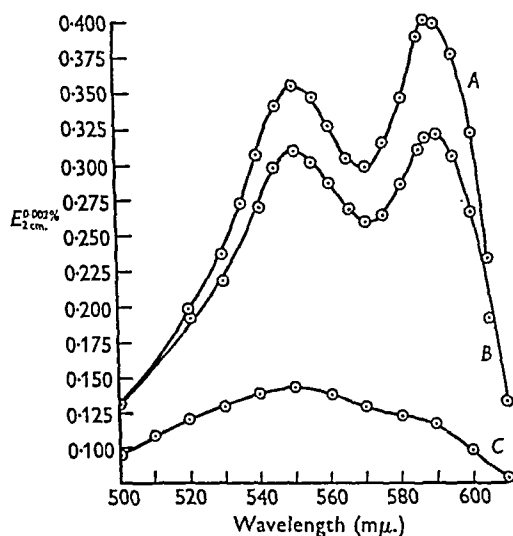


Fig. 4. Absorption spectrum of coloured complex derived from *N*-acetylglucosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after A, 1.5 hr., B, 4 hr. and C, 24 hr.

Effect of concentration of NaCl on the colour obtained. To a series of tubes each containing *N*-acetylglucosamine (20 μg . in 0.1 ml.) and 0.2 ml. of 0.125*N*- Na_2CO_3 , was added (a) water, (b) 1% NaCl, (c) 3% NaCl, (d) 5% NaCl or (e) 10% NaCl to yield a total volume of 1.1 ml. The resulting solutions were then treated by the standard procedure and the intensity of the colours finally obtained were read on the photoelectric colorimeter 0.5, 1, 1.5, 2 and 24 hr. after the addition of the DMAB reagent. The concentrations of NaCl used in the experiments (b) and (c) gave rise to no appreciable change in the rate or amount of colour obtained as compared with that which arises using the 'standard' conditions. The colour development in the presence of the NaCl concentrations (d) and (e) was slower, and the maximum colour intensity obtained was less.

Absorption spectrum of the *N*-acetylhexosamines after alkali treatment and the addition of DMAB reagent

Amounts of *N*-acetylglucosamine (20 μg .) and *N*-acetylchondrosamine (100 μg .), selected so that solutions of approximately the same colour intensity would finally result, were treated according to the standard procedure and, after standing for 1.5 hr. at 20°, examined in the spectrophotometer over the wavelength range 350–610 $\text{m}\mu$. Control solutions were treated in the same manner except that the *N*-acetylhexosamine and alkali mixture was not heated and no colour developed. The results are given in Figs. 4 and 5 and show that the *N*-acetylhexosamines give rise to a coloured complex with *p*-dimethylaminobenzaldehyde which shows light absorption with maxima at 550 and 590 $\text{m}\mu$. and a minimum at about 570 $\text{m}\mu$. No absorption peaks were observed between 350 and 500 $\text{m}\mu$. and this part of the curve is omitted in Figs. 4 and 5.

The rate of development and fading of the colour was also followed, and it was observed that the maximum intensity of

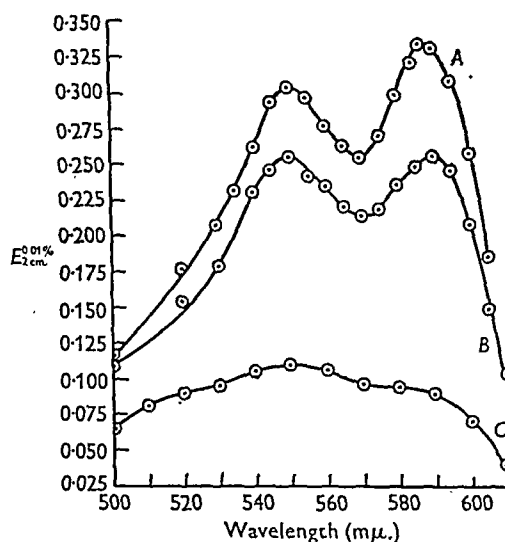


Fig. 5. Absorption spectrum of coloured complex derived from *N*-acetylchondrosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after A, 1.5 hr., B, 4 hr. and C, 24 hr.

absorption of both peaks (550 and 590 $\text{m}\mu$.) was reached at 20° in about 1.5 hr. after the addition of DMAB reagent. On standing the colour fades, but the rate of fall in the absorption intensity at 590 $\text{m}\mu$. was faster than that at 550 $\text{m}\mu$. The absorption intensity observed after the coloured solutions had stood for 4 hr. and 24 hr. for each *N*-acetylhexosamine is given in Figs. 4 and 5.

The two absorption peaks could be due to the interaction of one complex chromogenic structure or of two distinct chromogens with the *p*-dimethylaminobenzaldehyde. In order to determine if one of the absorption maxima arises from the presence of an intermediate product of the action of alkali on *N*-acetylglucosamine, specimens of this substance were heated at 100° for 1, 2, 4, 8 and 12 min. with 0.1 ml. of 0.5*N*- Na_2CO_3 , treated with glacial acetic acid and DMAB reagent in the usual manner and allowed to stand for 1.5 hr.

at 20°. The absorption curves obtained for a selected number of heating times, Fig. 6, demonstrate the similarity of the absorption after the different periods of heating, and show that the extent of heat treatment is reflected equally in the intensity of the absorption at 550 and at 590 $m\mu$. Similar results were obtained with *N*-acetylchondrosamine.

The coloured products obtained by heating *N*-acetylglucosamine and *N*-acetylchondrosamine with different buffer systems at 100°, such as with potassium borate (pH 11) or glycine buffer (pH 10.9) in place of Na_2CO_3 , and subsequent treatment with the DMAB reagent, have the same absorption characters. In order to obtain a maximum colour with these buffers, however, it was necessary to heat the borate and glycine buffer systems for 7 and 40 min., respectively, in place of the 4 min. heating required when Na_2CO_3 was employed (see Table 1).

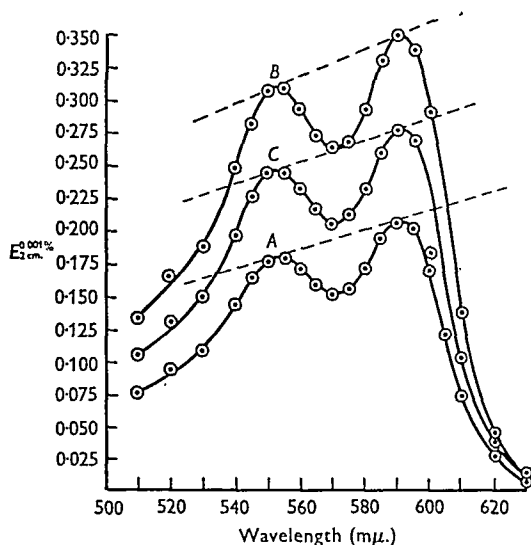


Fig. 6. Effect of varying the time of heating *N*-acetylglucosamine with Na_2CO_3 on the absorption spectrum of the coloured complex formed with DMAB. A, 2 min. heating; B, 4 min. heating; C, 8 min. heating.

The green filter used in the photoelectric colorimeter employed for the routine measurement of the intensity of the colour in the quantitative determination of the *N*-acetylhexosamines gives a maximum transmission of 42% at 550 $m\mu$. whilst at 570 and 590 $m\mu$. the transmissions are about 34 and 20%, respectively. Thus the filter measures predominantly the intensity of absorption at 550 $m\mu$.

The exact amount of colour given by *N*-acetylchondrosamine in terms of *N*-acetylglucosamine was determined by measuring the colour developed from a series of standard amounts of *N*-acetylglucosamine (50, 60, 70 and 80 μg .) and from 300 μg . of *N*-acetylchondrosamine. Measurements were made on the Uvispek spectrophotometer at 550 and 590 $m\mu$. using cells 2 cm. deep. The results showed that at 550 $m\mu$. 71 \pm 2 μg . of *N*-acetylglucosamine gave a solution of the same colour intensity as 300 μg . of *N*-acetylchondrosamine. At 590 $m\mu$. 68 \pm 2 μg . of *N*-acetylglucosamine were equivalent to 300 μg . of *N*-acetylchondrosamine.

Ultraviolet absorption of the *N*-acetylhexosamines after heating with alkali

N-Acetylglucosamine (300 μg . in 3 ml. water) was run into a 15 ml. volumetric flask, 1.5 ml. of 0.5N- Na_2CO_3 was added and the contents immediately made up to 15 ml. Half of the solution was heated for 4 min. at 100° and cooled; the remainder was unheated. The solutions were examined at once in the Uvispek spectrophotometer over the wavelengths 215–270 $m\mu$., the unheated portion of the solution, which itself showed a strong absorption at 217 $m\mu$., being used as the reference solution. The results are given in Fig. 7 and show that there is only a single absorption maximum and that this occurs at about 230 $m\mu$. The intensity of absorption at this wavelength is greatest after 4 min. heating, that is, after the time of heating necessary to yield subsequently the maximum colour following the addition of the DMAB reagent. The intensity of the absorption at 230 $m\mu$. is directly proportional to the amount of

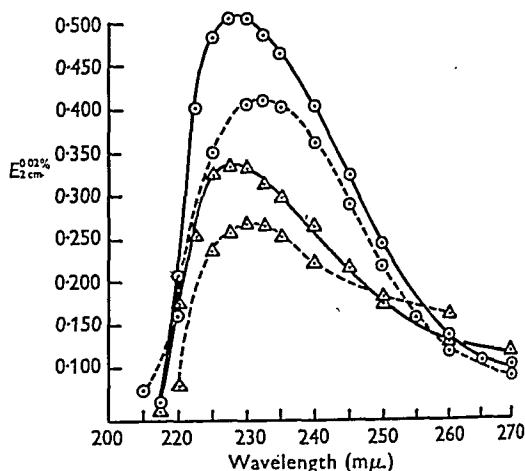


Fig. 7. Absorption in the ultraviolet region of *N*-acetylglucosamine and *N*-acetylchondrosamine after heating with Na_2CO_3 . *N*-Acetylglucosamine, \bigcirc — \bigcirc , after about 20 min.; \bigcirc — \cdots — \bigcirc , after 4 hr. *N*-Acetylchondrosamine, \triangle — \triangle , after about 20 min.; \triangle — \cdots — \triangle , after 4 hr.

N-acetylglucosamine heated with alkali and decreases on standing, with a slight shift in the position of the absorption maximum toward a longer wavelength. The absorption recorded after the alkali-treated *N*-acetylhexosamine solution had stood for 4 hr. at room temperature is also shown in Fig. 7. It is evident that the chromogenic structure is alkali-labile. The immediate addition of the DMAB reagent after the optimal time of heating with alkali is essential if maximum colour production is to be obtained subsequently in the colorimetric determination.

N-Acetylchondrosamine treated with dilute Na_2CO_3 under conditions identical with those described gives a similar absorption curve (Fig. 7) which likewise shows a maximum absorption at about 230 $m\mu$. It is to be noted, however, that the maximum intensity of the absorption at 230 $m\mu$. for *N*-acetylchondrosamine was considerably less than that given by an equal quantity of *N*-acetylglucosamine.

Solutions (20 pp./ml.) of methyl β -D-glucopyranoside, N-methyl β -glucosamine hydrochloride, β -tolyl β -D-glucosamine, N-acetylglucosamine, methyl N-acetylglucosamine, β -fructose, β -glucose, β -galactose, β -lactose, β -lactulose, β -fructulose, lactose, N-acetyl β -D-glucosamine and β -fructose were treated with alkali under the same conditions, but none of these substances gave an absorption curve similar to that obtained with the N-acetylhexosamines with a multiple maximum absorption at 210 m μ .

The absorption spectrum of glucose-lysine mixtures after alkali treatment and after alkali treatment and the addition of DMAB reagent

The finding by Vaesens & Immers (1949) that mixtures of certain amino acids, especially lysine, and simple sugars develop, after treatment with alkali and DMAB reagent, a colour similar to that given by the N-acetylhexosamines, suggested that the visible and ultraviolet absorption characteristics of lysine-glucose mixtures should be examined.

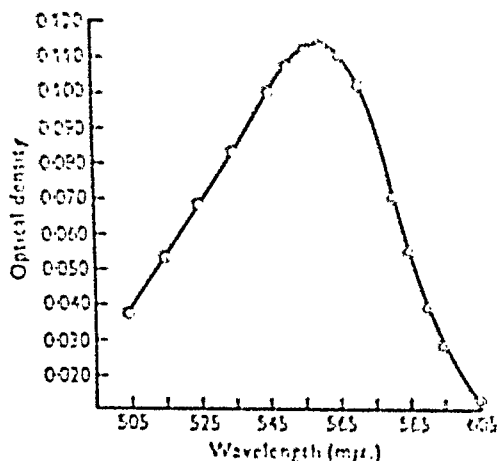


Fig. 8. The absorption spectrum of a mixture of glucose and lysine after treatment with Na_2CO_3 and DMAB.

Glucose (30 mg.) and lysine (15 mg.) were added separately and together to 1.5 ml. quantities of 0.5N Na_2CO_3 and the total volume in each instance was immediately made up to 15 ml. with water. The three solutions were divided into equal parts and one half of each was heated for 4 min. at 100° and then cooled. The solutions of glucose and of glucose + lysine developed a yellow colour, the solution of lysine remained colourless. The ultraviolet absorption of the solutions was then determined using the unheated portions as reference solutions. The results indicated that only the heated glucose and the heated glucose-lysine mixture showed any appreciable general absorption and in both instances the characteristic absorption maximum at 230 m μ . given by the N-acetylhexosamines was absent.

The heated and unheated test solutions (1 ml. of each) were then treated with glacial acetic acid and DMAB reagent (1 ml.) according to the usual procedure. The yellowish brown coloration of the reaction mixtures which contained heated glucose and glucose-lysine mixture disappeared on the addition of glacial acetic acid. The heated and unheated glucose and lysine solutions and the unheated glucose-lysine mixture developed a pale-yellow

colour only, whereas the heated glucose-lysine mixture (the only mixture developed a pink coloration). The absorption curves of the colour mixtures were given in Fig. 9 and show only a small absorption maximum at 210 m μ .

Alkali alone. The blood-group substances A, B, H and L α were treated with alkali and DMAB reagent and the results obtained are given in Fig. 10.

The blood-group substances A, B, H and L α were treated with alkali and DMAB reagent and the results obtained are given in Fig. 10. The blood-group substances A, B, H and L α were treated with alkali and DMAB reagent and the results obtained are given in Fig. 10.

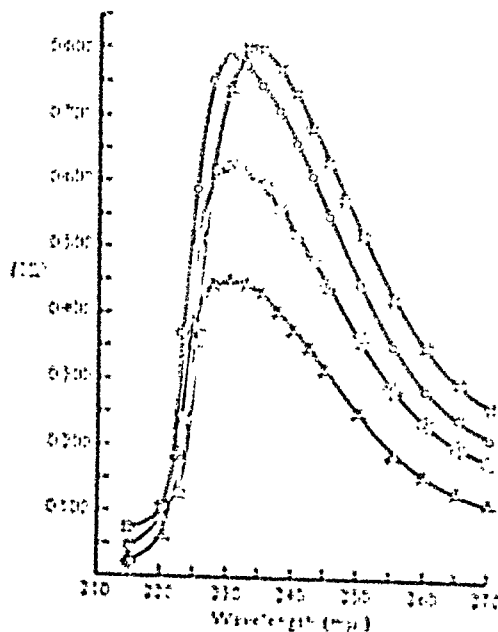


Fig. 9. Absorption in the ultraviolet of blood-group mucoids after treatment with dilute alkali at 100°C . \bigcirc — \bigcirc , A substance; Δ — Δ , B substance; \times — \times , H substance; \bullet — \bullet , L α substance.

the observations of Vaesens & Immers (1949) that several component molecules of the group mucoids other than the N-acetylhexosamines, such as galactose and fructose and the individual amino-acids, especially if free, will combine in alkaline solution to form a substance which will give a coloured complex with DMAB. It became of interest, therefore, to examine not only the amount of colour given, but also the absorption characteristics of the alkali-treated blood-group substances and coloured products obtained from them on the addition of the DMAB reagent.

Alkali alone. The group substances A, B, H and L α (3 mg. in 1.5 ml. 0.5N Na_2CO_3) were each made up to 15 ml. with water and divided into two approximately equal parts. One part was heated at 100° for 12 or 15 min. (see below), whilst the remainder of the solution was kept as an unheated control. The heated solution was cooled and examined immediately in the spectrophotometer, and its absorption with reference to the unheated material was measured over the wavelength range 215–270 m μ . The results obtained are given in Fig. 9 and show that in each

instance a structure is formed which gives a single absorption maximum at about 230 mμ. Solutions of equal concentration (200 μg./ml.) of dextran, glycogen, gum arabic, manncarolose, galactocarolose, and the specific polysaccharides of *Shigella shigae* and pneumococcus Type II gave no comparable amount of absorption over the same wavelength range and showed no characteristic absorption maximum at 230 mμ. after treatment with alkali under the same conditions as were used for the group mucoids.

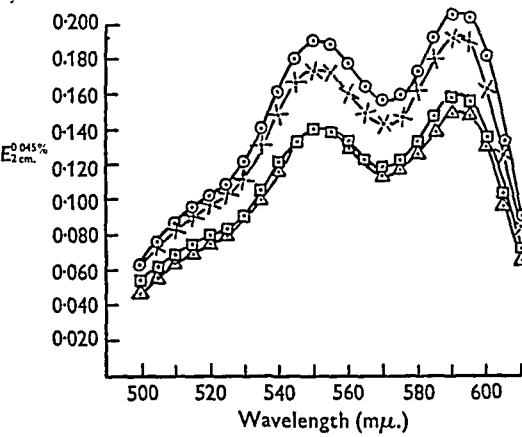


Fig. 10. Absorption spectrum of coloured complex derived from blood-group mucoids. ○—○, A substance; □—□, B substance; ×—×, H substance; △—△, Le^a substance.

Alkali and DMAB reagent. The group substances (500 μg.) contained in 1 ml. water were heated with 0.1 ml. of 0.5N-Na₂CO₃ for 12 or 15 min. (see below), cooled and treated according to the standard procedure. The absorption spectrum of each group substance was measured over the range 500–610 mμ. using the unheated group substance, which in no instance gave a purple colour under the test conditions, as the reference solution. Group mucoids so treated sometimes develop a slight opalescence which should be removed by the addition of 1 ml. water to all solutions just before the absorption intensities are measured. The results are given in Fig. 10 and indicate that absorption

curves similar to those given by the *N*-acetylhexosamines were obtained. The points of maximum absorption intensity were again at about 550 and 590 mμ. with a minimum at 570 mμ.

The influence of the time of heating of the group mucoids with alkali on the intensity of the colour subsequently obtained on the addition of the DMAB reagent was investigated using the 'standard' procedure, and the results obtained (Table 2) in one instance (A substance) showed that the optimal period of heating is 12 min. Somewhat longer times, about 15 min., were required by B and H substances. It seems that a minimum time of 12 min. heating should be employed when measuring the '*N*-acetylhexosamine' colour given by the group mucoids. The polysaccharides used as control materials and mentioned above give no colour after heating with alkali for 15 min. and the addition of the DMAB reagent.

The amount of colour obtained with A substance, after treatment with dilute Na₂CO₃ or one of the other buffers, is recorded in Table 2 where the results are expressed in terms of the absorption given by an equal weight of *N*-acetylglucosamine. The values obtained may be compared with those given for *N*-acetylglucosamine or *N*-acetylchondrosamine (Table 1) and show that heating the A substance with borate buffer gives rise to a material which shows about the same intensity of absorption as is obtained with glycine, or Na₂CO₃, in contrast to the behaviour of the *N*-acetylhexosamines which give an enhanced colour when heated in borate buffer. The group substances give maximum colour intensities equivalent to the absorption given by 7–10% of their weight of *N*-acetylglucosamine.

The influence of oxidation of A substance on the absorption spectrum

Oxidation with sodium periodate. A maximum intensity of absorption was observed at 230 mμ. with A substance oxidized with periodate at pH 5 for 7 hr. (Aminoff & Morgan, 1951) and treated with alkali alone. The addition of the DMAB reagent gave rise to the production of a purple colour which showed two absorption maxima, at 550 and 590 mμ. A quantitative determination of the amount of '*N*-acetylglucosamine' colour given by the oxidized A substance showed that there was probably no significant

Table 2. Amount of colour given by blood-group A substance after treatment at 100° with different buffer systems and the addition of DMAB reagent

Alkaline system	pH	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine*	
			(Observed)	(Extrapolated value)
0.1 ml. 0.125N-Na ₂ CO ₃	10.8	35	7.8	9.2
0.4 ml. 0.125N-Na ₂ CO ₃	10.8	12	6.8	9.1
0.8 ml. 0.125N-Na ₂ CO ₃	10.8	10	8.4	10.7
Potassium borate + KOH	9.0	†	—	—
	10.0	30	6.2	7.1
	11.0	45	6.2	7.9
Glycine buffer	10.0	†	—	—
	10.9	150	7.1	8.5
	12.2	5	6.2	7.9

* Under 'standard' conditions. † Maximum colour not reached after 4 hr. heating.

change in this property as a result of oxidation with periodate. In these respects, therefore, the oxidized A substance behaves as does the untreated blood-group substance.

Oxidation with hypiodite. The procedure described by MacLeod & Robison (1929) was used. A substance (5 ml. of 0.5%) was added to 3.0 ml. of 0.1 *N*-I₂ and 2.0 ml. of 0.5 *N*-Na₂CO₃ contained in a 50 ml. glass-stoppered flask. The solution was kept at 22° in the dark for 7 hr., after which time oxidation was essentially complete and the serological activity of the A substance was largely destroyed. The oxidized mucoid was dialysed against distilled water at 0°, made up to a known volume and 500 μ g. were treated with alkali according to the conditions described in the standard procedure for the determination of *N*-acetylglucosamine. The oxidized and alkali-treated material showed a maximum absorption at 230 $m\mu$., but the intensity of absorption was less than that given by an equal quantity of unoxidized material. The addition of the DMAB reagent gave rise to a purple-coloured solution which showed two absorption maxima, at 550 and at 590 $m\mu$. A quantitative measurement of the '*N*-acetylglucosamine' colour revealed that the A substance after oxidation with hypiodite gave rise to about 80% of the colour intensity reached by the original A substance. A detailed account of the oxidation of the blood-group mucoids with hypiodite will be given elsewhere.

DISCUSSION

The conditions usually employed for the colorimetric determination of the *N*-acetylhexosamines have been re-examined in some detail, and it is evident from the results obtained that these substances undergo rapid structural change on treatment for a short time with hot dilute alkali or alkaline buffers, and that in each instance a substance is formed which shows strong light absorption between 220 and 250 $m\mu$. with a maximum absorption at about 230 $m\mu$. The gradual disappearance of the absorption maximum observed, however, indicates that the substance formed by the action of alkali is itself unstable in alkaline solution.

The substances formed from the *N*-acetylhexosamines by the action of dilute alkali condense readily with *p*-dimethylaminobenzaldehyde in acetic acid to yield a solution of intense purple colour which shows characteristic absorption maxima at 550 and 590 $m\mu$. with a minimum at about 570 $m\mu$. (cf. Osaki & Turumi, 1947; Gottschalk & Lind, 1949). In view of the unstable nature of the chromogenic material, it is essential for the quantitative determination of *N*-acetylhexosamine that the *p*-dimethylaminobenzaldehyde reagent should be added and the test completed immediately the solutions under examination have been heated with alkali.

Treatment of the *N*-acetylhexosamines with alkali under different conditions has revealed that increasing the concentration of Na₂CO₃ results not only in a more rapid formation of the chromogenic structure, but also brings about its more rapid destruction and, although the period of heating

necessary to yield the maximum colour after the addition of the *p*-dimethylaminobenzaldehyde reagent becomes shorter with increasing alkali concentration, the actual amount of colour obtained decreases with higher concentration of alkali. Extrapolation of that part of the curve which measures the rate of destruction of the chromogenic material gives a corrected value for the maximum colour intensity found. The corrected value obtained, however, is of no great accuracy. It will be noticed that the alkali concentration finally recommended as suitable for the quantitative determination of the *N*-acetylhexosamines is not that which gives rise to the minimum destruction of the chromogenic substances and consequently to greatest colour intensity per unit weight of the *N*-acetylhexosamines. The alkali concentration finally chosen was selected so that any slight buffer action of the solution under test would not alter significantly the pH of the solution during treatment with alkali which should be close to pH 10.8. Treatment of the *N*-acetylhexosamines with borate buffer at pH 10.8 gives rise to an enhanced colour production as compared with that obtained using sodium carbonate at the same pH.

The interaction in an alkaline medium of simple hexoses and certain amino-acids with the formation of a substance that gives with *p*-dimethylaminobenzaldehyde reagent a colour which closely resembles that given by *N*-acetylglucosamine after similar treatment was first reported by Vasseur & Immers (1949) and subsequently confirmed by Gottschalk & Partridge (1950). The light absorption given by a mixture of lysine and glucose after treatment with dilute alkali has now been determined, and it has been found that within the range 210–300 $m\mu$. there is no characteristic absorption maximum similar to that given by the *N*-acetylhexosamines. The purple-coloured solution which is formed on the addition of the *p*-dimethylaminobenzaldehyde reagent to the alkali-treated glucose-lysine mixture shows a single absorption maximum at 560 $m\mu$. and not two maxima at 550 and 590 $m\mu$. as is shown by the *N*-acetylhexosamines after identical treatment. The product of the interaction of glucose and lysine, therefore, although giving a reddish purple colour with *p*-dimethylaminobenzaldehyde is nevertheless readily distinguished from that which arises from the *N*-acetylhexosamines. The colour given in the 'Direct Test' of Osaki & Turumi (1947) appears to be due to an amino-acid-sugar complex and not, as they suggest, to a different ring structure associated with *N*-acetylhexosamine.

Morgan & King (1943) reported that a purified mucoid which was obtained from hog gastric mucin and which possessed intense human blood-group A activity, gave a colour reaction after treatment with

dilute alkali and *p*-dimethylaminobenzaldehyde similar to that given by *N*-acetylglucosamine. Subsequently it was suggested (Morgan & Waddell, 1945; Morgan, 1947) that this colour reaction was a characteristic property of the blood-group mucoids. It is now known that solutions of essentially homogeneous preparations of the blood-group mucoids which are responsible for the group characters A, B, the recently discovered 'Lewis' Le^a property and the so-called O (H) character of the tissue fluids and secretions, all develop a purple colour after treatment with dilute alkali and the addition of the *p*-dimethylaminobenzaldehyde-hydrochloric acid reagent. In each instance the maximum light absorption of the coloured solution is found to be at about 550 and 590 m μ ., whereas the solutions of the material after alkali treatment alone show a single absorption maximum at 230 m μ . In both these respects, therefore, the group substances behave as do the *N*-acetylhexosamines.

The blood-group substances require a minimum of about 12 min. heating with alkali to yield the maximum amount of colour with the *p*-dimethylaminobenzaldehyde reagent in place of the 4 min. heating necessary for the *N*-acetylhexosamines. The intensity of the colour given by the group substances under these conditions varies somewhat for each group substance investigated, but is found to be equivalent to between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine. It is to be noted that there is no enhancement of colour production when a borate buffer of the same pH value is employed.

Group A substance rendered serologically inactive by oxidation with sodium periodate at pH 5 continues to yield a strong purple colour after treatment with alkali and the addition of DMAB reagent (Aminoff & Morgan, 1951). It is now shown that A substance after oxidation with periodate and treatment with alkali shows a single absorption maximum at 230 m μ . and that the purple colour which subsequently arises after the addition of the DMAB reagent also shows absorption maxima of undiminished intensity at 550 and 590 m μ . The A substance after oxidation with periodate therefore behaves as does the unoxidized material. Aminoff & Morgan (1951) considered that oxidation of the A substance with periodate left unchanged the reducing group and the *N*-acetyl group of the *N*-acetylchondrosamine end unit, to allow for the formation of the chromogenic structure on treatment with alkali, and the results of the absorption studies now reported with A substance oxidized with periodate appear to support this suggestion.

Oxidation of the A substance with sodium hypiodite, on the other hand, forms a substance which gives a reduced '*N*-acetylglucosamine' colour

value after alkali treatment and the addition of *p*-dimethylaminobenzaldehyde. It seems probable that oxidation of the A substance with hypiodite converts the aldehyde group of the *N*-acetylchondrosamine residue present as a reducing end group into a carboxyl group. Such a change would result in the end residue being unable to form a chromogenic structure on treatment with alkali and in consequence the oxidized A substance would yield with *p*-dimethylaminobenzaldehyde a solution of diminished colour intensity. As *N*-acetylchondrosamine gives about one-fifth of the amount of colour given by *N*-acetylglucosamine it must be concluded that *N*-acetylglucosamine contributes to the total colour given by the A substance. Aminoff & Morgan (1949, 1951) suggested that the terminal *N*-acetylchondrosamine residue is linked to C atom 1 of its neighbouring *N*-acetylglucosamine residue and that treatment of the A substance with alkali under the conditions used for the determination of *N*-acetylhexosamine hydrolyses this glycoside linkage and thus transforms the penultimate *N*-acetylglucosamine into a reducing end group and renders it susceptible to the action of alkali with the consequent formation of a chromogenic structure which gives a coloured complex with *p*-dimethylaminobenzaldehyde (cf. Morgan, 1946). If the total colour given by the A substance after treatment with alkali and *p*-dimethylaminobenzaldehyde arises in this way from *N*-acetylchondrosamine and *N*-acetylglucosamine units then the decrease in the colour intensity to be expected from A substance after treatment with hypiodite will be about 18 % of the original value expressed in terms of *N*-acetylglucosamine. A decrease of this order has been found. The action of alkaline hypiodite and of dilute alkali on such complex materials as the blood-group mucoids is, however, far from straightforward, and the results of further work must be awaited before the changes brought about can be fully understood or the results obtained used to establish the presence of definite structures within the blood-group mucoids.

SUMMARY

1. The conditions for the colorimetric determination of the *N*-acetylhexosamines have been re-investigated.

2. The period of heating necessary, and the maximum amount of colour obtained, when *N*-acetylglucosamine is treated with alkali and the *p*-dimethylaminobenzaldehyde reagent, depends not only on the pH of the system but also on the nature of the buffer employed. Borate buffer gives an enhanced colour production.

3. *N*-Acetylchondrosamine behaves similarly, but the intensity of the colour given is about 23 %

of that obtained with an equal weight of *N*-acetylglucosamine.

4. The *N*-acetylhexosamines after treatment with alkali show a single maximum absorption at 230 m μ . and after the addition of the *p*-dimethylaminobenzaldehyde reagent the purple colour which develops shows two absorption maxima, at 550 and at 590 m μ .

5. A mixture of glucose and lysine heated with sodium carbonate shows no characteristic absorption maximum at 230 m μ . The purple colour obtained on the subsequent addition of *p*-dimethylaminobenzaldehyde reagent shows a single absorption maximum at about 560 m μ .

6. The human blood-group mucoids yield between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine, and there is no enhanced colour production when a borate buffer is employed. The absorption characteristics of the chromogenic substances which arise from the group substance after heating with alkali, and of the coloured product obtained on the addition of the

p-dimethylaminobenzaldehyde reagent support the belief that the colour given by the group substances arises from reducing *N*-acetylchondrosamine end groups and closely linked *N*-acetylglucosamine residues, which are readily liberated by the action of alkali.

7. The A substance, after oxidation with hypiodite, gives rise to a coloured complex on treatment with alkali and *p*-dimethylaminobenzaldehyde which shows a reduced absorption intensity compared with that given by the unoxidized material. A substance oxidized with periodate and treated under similar conditions shows no decrease in absorption. The significance of these findings is discussed.

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A Study of the Breakdown of Ribonucleic Acid in Tobacco-leaf Extracts

By G. PARKER*

Rothamsted Experimental Station, Harpenden, Herts

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There is an extensive literature on the preparation of ribonucleic acids and their degradation products from animal tissues and micro-organisms. By contrast, apart from the isolation of certain plant virus nucleoproteins, little work has been undertaken until recently, on the preparation of ribonucleic acids from plant material.

* Present address: The Distillers Co. Ltd. Speke, Liverpool 19.

Foreman (1938) suggested the presence of nucleoprotein in leaves of perennial rye grass, to explain the fact that cytolysed leaves yielded only 30 % of their phosphorus on extraction with boiling water, whereas leaves previously dried at 85° yielded all their phosphorus by cold water extraction. The occurrence in, and isolation of, ribonucleic acid from leaves of barley, rye and spinach has been reported by von Euler & Hahn (1947, 1948). These

workers used an alkaline extraction method similar to that employed by Schmidt & Thannhauser (1945) for animal tissues. Takasugi (1943, 1944) used 10% sodium chloride solution to extract the nucleic acids from barley roots. Ogur & Rosen (1950) used perchloric acid for the separate extraction of ribo- and deoxyribo-nucleic acids from corn root tips.

Pirie (1950) has described the isolation of a ribo-nucleoprotein from the sap of the leaves of healthy tobacco plants. As prepared, this material, whose concentration in sap varies with the age of the leaf, is associated with enzymes (ribonuclease and phosphatase) capable of bringing about its degradation. Pirie was able to obtain the nucleic acid from fresh preparations of this nucleoprotein by denaturation of the protein with trichloroacetic acid (TCA), but he could only isolate degradation products of the acid after such precipitation from sap which had not been ultracentrifuged. It appeared of interest to examine these degradation products more closely, and the work reported in the present paper deals with the preparations of various phosphorus-containing fractions obtained from sap by chemical and enzymic degradation of the nucleic acid moiety of the trichloroacetic acid sap precipitate which contains this nucleoprotein.

MATERIALS AND GENERAL PROCEDURES

Preparation of sap-trichloroacetic acid precipitate and of chloroplast-trichloroacetic acid precipitate

Leaves, between 10 and 20 cm. long, of tobacco (*Nicotiana tabacum* var. White Burley) from plants grown in a heated glasshouse were used for most of the work. Single experiments confirming the results obtained with tobacco have also been carried out with the leaves of turnip, spinach, French bean, comfrey, snowberry and grass, and oat and barley seedlings. Unless stated otherwise, results quoted in the text refer to tobacco. The leaves were taken at all periods of the year; different batches consequently probably differed in age, but no attempt has been made to analyse the results for possible seasonal variations in composition.

The midribs were removed from the leaves and the laminae passed through a domestic mincer and squeezed on madapolam into a receiver cooled in ice. The residue was re-minced and squeezed again. Cell debris, chloroplasts and starch granules were removed by centrifuging for 15 min. in a chilled angle centrifuge at 8000 rev./min. (6400g). The supernatant fluid, which is subsequently referred to as sap, was stored at 4° and used within 30 min. of preparation. Unless otherwise stated, it was treated with 50% (w/v) trichloroacetic acid (TCA) to a final concentration of 5%. The precipitate was centrifuged down at once and washed several times, first with 2% (w/v) TCA and then with water, the volume of wash liquor in each case being about half that of the sap, until the washings contained only 1–2 µg. P/ml. The solid residue is subsequently termed sap-TCA precipitate.

The chloroplasts, separating from crude sap as a sticky layer overlying the starch, were roughly separated from the latter by scraping with a spatula. They were washed twice with distilled water and were then dispersed to give a suspension which was used immediately after preparation. It was precipitated with TCA and washed in the same way as the sap, and is referred to subsequently as chloroplast-TCA precipitate.

Analytical methods

Phosphorus. This was determined by a modification of the method of Kuttner & Lichtenstein (1932). Inorganic phosphate values were obtained on samples which had not been incinerated. P retained by samples containing Ba was estimated by incineration with H₂SO₄ until the sample dissolved completely: after dilution, the precipitate was centrifuged down, and the inorganic phosphate content of the supernatant liquid measured.

Ribose. Total ribose estimations were made by the orcinol method of Pirie (1936) using ribose standards. The colour developed was measured in a photoelectric absorptiometer (the Evans Electroselenium Portable model was used) using Ilford filter 622.

Values for purine-bound ribose were obtained by the Markham & Smith (1949) modification of Miltzer's (1946) orcinol method. The colour developed was read in the absorptiometer against that due to ribose standards, using Ilford filter 205.

Purines. These were determined in N-H₂SO₄ hydrolysates by the following methods.

Adenine. This was estimated colorimetrically by Woodhouse's (1950) method, modified by the use of twice the concentration of ammonium sulphamate recorded by Woodhouse. This avoided difficulties, otherwise encountered, through incomplete decomposition of excess nitrite, which led to production of cloudy solutions.

Guanine. The method of Williams (1950) was employed. Standard guanine solutions were subjected to precipitation along with the samples, and the colour developed with the phenol reagent was read, using Ilford filter 608.

Total pyrimidines. These were obtained in the free form by the method of Daly, Allfrey & Mirsky (1950), and were then determined by the colorimetric procedure of Soodak, Pircio & Cerecedo (1949) using standard uracil solutions and Ilford filter 608.

Phosphatase activity. This was determined using, as substrate, solutions of sodium β-glycerophosphate and metaphosphate, and yeast adenylic acid. The reaction mixture comprised substrate solution at pH 6, 0.02M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total volume of 5 ml., and contained 100 µg. P/ml. It was incubated at 37°. Samples were withdrawn at intervals and pipetted into an equal volume of 10% (w/v) TCA: after centrifuging at 1500 rev./min. for 10 min., the supernatant fluid was poured off and analysed for inorganic phosphate. Control reaction mixtures, from which the enzyme preparations and the substrate were individually omitted, were set up initially. They were found to give such low blank values as to be unnecessary, and were omitted in the later experiments.

Ribonuclease activity. This was measured by determining the amount of P becoming soluble in the uranyl nitrate and TCA reagent of McFadyen (1934) after action of the enzyme preparation on yeast ribonucleic acid. The reaction mixture comprised a solution of the acid brought to pH 6, purified by

the method of Vischer & Chargaff (1948b), 0.02M-sodium citrate buffer, pH 6, enzyme solution or suspension, and water to a total vol. of 5 ml., and contained 100 μ g. P/ml. It was incubated at 37°. Samples were removed at intervals into 1 ml. of the uranyl reagent, chilled at 0° for 15 min. and then centrifuged at 1500 rev./min. for 10 min.; the supernatant fluid was poured off and an appropriate volume taken for total P analysis. As in the case of the phosphatase determinations, control reaction mixtures omitting enzyme or substrate were found to be unnecessary, and were only set up in a few preliminary experiments.

The optimum pH for nuclease activity was obtained in a similar reaction mixture, using the Michaelis (1931) veronal-acetate buffer (1.0 ml. of the diluted stock solution in 5 ml. total reaction mixture) of appropriate pH. The pH of the reaction mixture was determined at the beginning and the end of the experiment, but showed no signs of drift during the reaction at any pH employed.

Paper chromatography. Purines and pyrimidine nucleotides were separated on Whatman no. 1 or no. 11 paper using the *tert.*-butanol-HCl solvent system of Smith & Markham (1950). They were located on the paper by the mercury reagents of Vischer & Chargaff (1948a) and by viewing in suitably filtered ultraviolet light (Holiday & Johnson, 1949).

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph.

pH measurements. These were made with a glass electrode.

RESULTS

Fractionation of sap-TCA precipitate: preparation and properties of mononucleotide solutions

Sap-TCA precipitate, prepared as described, was mixed with water to a thin paste, cooled to 0° and brought to pH 9 with 0.5N-NaOH. Partial solution occurred. After standing overnight at 4°, the mixture was centrifuged at 8000 rev./min. (6400g) and the supernatant decanted. The residue was re-extracted with NaOH, pH 9, at 4° for several hours, again centrifuged down and then washed with a little water. The combined dark brown extracts, the total volume of which was approximately one-third that of the original sap, contained much of the protein originally present in the sap-TCA precipitate. This was thrown out by progressive acidification to a pH of approximately 6, 5 and 4. At each pH the precipitate was centrifuged down and washed with a little water, the washings being added to the main supernatant before the pH was readjusted. The almost colourless pH 4 supernatant contained no nucleic acid as it gave no precipitate on acidifying to pH 1 or less. It was treated with saturated Ba(OH)₂ solution to pH 9 and stood overnight at 4°. Any precipitate separating was centrifuged out, and the nucleotides were precipitated from the supernatant with 3 vol. of 95% ethanol at 4°. The barium salts were dissolved in the minimum volume of water and decomposed with 0.5N-H₂SO₄ to pH 3. Precipitated BaSO₄ was centrifuged down and washed with a little water, and the combined supernatants were treated with a little Ba(OH)₂ solution and quantitatively freed from Ba⁺⁺ ions with 5% (w/v) Na₂SO₄ before finally centrifuging clear. The aqueous ethanolic supernatant remaining after separation of the barium nucleotides usually yielded a second crop of barium salts when treated with more Ba(OH)₂ solution and stored at 4°

for 24 hr.: these were decomposed with H₂SO₄ in the same way.

In Table 1 is given the distribution of P between the residue insoluble at pH 9, the nucleotide preparations and the protein precipitates in the above procedure. These three fractions comprise the bulk of the P originally present in the sap-TCA precipitate. The remaining 10% was mostly found

Table 1. *Distribution of phosphorus in some fractions of sap*

Fraction	P, as % of total of		
	Sap	Sap-TCA precipitate	pH 9 extracts
pH 9-insoluble residues	1-3	5-15	—
Mononucleotide preparations	7-10	40-55	50-65
Protein precipitates	3-8	20-35	20-40

associated with the BaSO₄ precipitates obtained by decomposition of the barium nucleotides. Small amounts were also found in the precipitates sometimes obtained with Ba⁺⁺ at pH 9 before addition of ethanol, and in the aqueous ethanolic supernatants remaining after precipitation of the 'second crop' nucleotides. In this way it is possible to account for a total of over 95% of the P of the sap-TCA precipitate.

In a single experiment, several of the aqueous ethanolic solutions remaining after precipitation of two crops of nucleotides were pooled and evaporated to one-tenth volume in vacuum, but attempted precipitation of further nucleotides by Ba(OH)₂ and ethanol yielded no baryta-soluble, ethanol-insoluble material.

The figures in Table 1 vary somewhat with each batch of sap. This is not surprising. Variations are to be expected from causes such as differences in age of leaves taken at various times of the year, the use of different samples of soil in growing the plants, and unavoidable variations in the experimental technique with different lots of leaves. The values given are the extreme ranges obtained with several different batches of sap.

No precipitate was produced in the preparations by solutions of uranyl nitrate in TCA at a pH of less than 1, but addition of alkali caused mononucleotides to be thrown down, maximum visible precipitation occurring in the range pH 1.5-2 (cf. Zittle, 1946). Treatment with picric acid had no effect, but Buell's (1943) aluminium picrate reagent gave immediate yellow precipitates containing the adenylic acid and most of the guanylic acid of the preparations. Pyrimidine nucleotides are not precipitated by this reagent but their occurrence was indicated by the presence of a substantial proportion (50-60%) of the initial P in the filtrates. Confirmation was obtained in later experiments, when paper chromatography of preparations after 60 min. hydrolysis in N-HCl showed the presence of the four components, adenine, guanine, cytidylic and uridylic acids, typical of ribonucleic acids.

Other experiments of the type described, all carried out on 'first crop' nucleotide preparations, indicated that these contained more pyrimidine than purine bases. This was supported by analysis of the preparations for purine-bound ribose, which indicated that this accounted for some 40% of the total carbohydrate. Confirmation was obtained by analyses of the preparations for purine and pyrimidine content. Typical results are shown in Table 2, which also

Table 2. *Purine and pyrimidine composition of two nucleotide preparations*

(In exp. 1, 1 l. sap contained 191 mg. P. It gave a sap-TCA precipitate containing 27.3 mg. P, and yielded 15.3 mg. P in nucleotide preparations. In exp. 2, the sap had 179 mg. P/l. and gave a sap-TCA precipitate containing 45.3 mg. P and yielded 17.7 mg. P in nucleotide preparations. 'First crop' indicates nucleotides obtained from the initial baryta-soluble, ethanol-insoluble precipitates; 'second crop' preparations resulted from addition of more Ba(OH)₂ to the supernatants from the 'first crop' precipitates. The composition of the nucleotide preparations is given as molecules base/100 atoms P.)

Exp.	Fraction	Adenine (molecules/100 atoms total P)	Guanine (molecules/100 atoms total P)	Total pyrimidines (molecules/100 atoms total P)	P (atoms)
1	First crop	2.2	32.8	58.9	93.7
	Second crop	0.6	4.6	1.2	6.3
	Total	2.8	37.4	60.1	100
2	First crop	1.5	26.7	65.0	90.4
	Second crop	3.1	5.1	1.6	9.6
	Total	4.6	31.8	66.6	100

Table 3. *Effect of removal of lipids upon yields and composition of nucleotide preparations*

(Two equal portions of sap, from the same batch of leaves, were precipitated with TCA. The sap-TCA precipitates were fractionated for nucleotides as described in the text, one being treated with lipid solvents before fractionation. 'First' and 'second' crops have the meanings designated in Table 2. The composition of the preparations is given as molecules base/100 atoms P. Results of experiments on two separate lots of leaves are presented: (1) the sap (1 l., 231 mg. P) gave 45.5 mg. P in the sap-TCA precipitate. If lipids were not removed 21.2 mg. P were recovered in nucleotide preparations; if lipids were removed before fractionation, only 14.7 mg. P were so recovered; (2) the sap (1 l., 183 mg. P) gave 37.8 mg. P in the sap-TCA precipitate. This yielded 16.5 mg. P and 13.6 mg. P respectively as nucleotides following fractionation in presence and in absence of lipids.)

Lipids present	Fraction	Adenine		Guanine		Total pyrimidines		Phosphorus	
		Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2	Sap 1	Sap 2
		(molecules/100 atoms total P)							
Lipids present	First crop	2.2	2.3	29.9	19.4	57.1	63.8	90.1	85.2
	Second crop	0.6	0.6	4.0	6.7	3.5	5.8	9.9	14.8
	Total	2.8	2.9	33.9	26.1	60.6	69.6	100	100
Lipids absent	First crop	0.4	0.2	32.5	30.4	42.7	52.8	78.2	86.9
	Second crop	3.9	1.7	10.5	6.4	7.0	4.9	21.8	13.1
	Total	4.3	1.9	43.0	36.8	49.7	57.7	100	100

shows that in the 'second crop' preparations this order is reversed and purines predominate. However, the total amount of pyrimidines in the 'first' and 'second' crops from any one batch of sap is always considerably greater than the total amount of purines.

Effect of ageing sap-TCA precipitate on phosphorus distribution

A small number of experiments was carried out on sap-TCA precipitates which had been left in contact with their TCA supernatants at room temperature for some hours before collection. The results were rather variable. However, the sap-TCA precipitate collected immediately after preparation usually contained somewhat more P than did the corresponding precipitate, from an equal volume of sap, allowed to age for some hours before it was collected. The difference increased the longer the precipitate was left to age. Aged precipitates yielded less P in the pH 9 extracts than did the fresh precipitates, but these extracts yielded protein precipitates which retained more P than did fresh sap-TCA precipitate extracts. Invariably, ageing of a sap-TCA precipitate led to reduction in the amount of P that was isolated in the nucleotide preparations.

Effect of removal of lipids from the sap-TCA precipitate upon yield and composition of the nucleotide preparations

Two equal volumes of a sap were precipitated with TCA and the precipitates washed in the usual manner. One precipitate was subjected to alkaline fractionation immediately. The other was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless; the residue was washed with water and then fractionated for nucleotides. Results of typical experiments are given in Table 3. This shows that removal of lipids from the sap-TCA precipitate lessened the amount of P which could be isolated as nucleotides. This was reflected in the total amount of pyrimidines present in the nucleotide preparations. The purine contents were affected to a very much smaller extent and the change may not be significant as it is difficult to ensure absolutely identical conditions of fractionation for each half of the batch of sap. The amount of P separating with the protein precipitates during fractionation was correspondingly greater in these cases than in the parallel experiments on precipitates from which the lipids were not removed. Removal of lipids from the sap-TCA

precipitate had no effect upon the amount of P which could be subsequently removed from the material insoluble at pH 9. This is described more fully in a subsequent section.

Further fractionation of pooled pH 9-insoluble residues and protein precipitates

In a few preliminary experiments, the residue remaining after two extractions of the sap-TCA precipitate was pooled with the protein precipitates and refractionated after a further TCA precipitation. Table 4 shows that some 70% of the P remained in the TCA supernatant. Most of the P precipitated was either not extracted at pH 9 or precipitated along with the proteins again, only 5–15% (i.e. 2–6% of the initial total) being finally obtained in nucleotide preparations.

Table 4. *Phosphorus distribution during fractionation of pooled pH 9-insoluble residues and protein precipitates*

(The figures refer to three separate fractionations of pooled material)

Percentage total P in:	
5% TCA supernatant	70.2, 71.4, 63.0
Nucleotide preparations	4.5, 1.9, 6.1
Percentage P of TCA precipitate in:	
pH 9-insoluble residues	38.6, 29.4, 51.6
Nucleotide preparations	14.9, 6.4, 11.5
Protein precipitates	25.2, 47.3, 22.7

Phosphorus content of pH 9-insoluble residues

The residue from sap-TCA precipitate after two extractions at pH 9 usually consisted of a gel which did not sediment cleanly at 8000 rev./min., and still contained 5–15% of the P of the sap-TCA precipitate. The material was difficult to mix effectively with extracting liquid, but 50–60% of its P could be removed by repeatedly washing with water (Table 5). If the material were given a preliminary treatment with 5% (w/v) TCA and then washed several times with water, a larger proportion (70–80%) was extracted. This is probably due to the change in texture of the material, on treatment with the TCA, to a hard, granular form, readily sedimenting at 1500 rev./min., for, in samples that had been washed several times with water until no more P was extracted, treatment of the residues with 5% (w/v) TCA liberated more P and in this way 70–80% of the original amount could again eventually be extracted.

Effect of incubation on the pH 9-insoluble residues

Increases in the amount of P that could be extracted by water from the pH 9-insoluble preparations, were observed on raising the temperature and extending the time of contact between the solvent and the sample. Lipid-free material incubated aseptically with water at 37° for 2–3 days yielded 80–90% of its P in water extracts. Samples thus treated did not liberate further P by subsequent treatment with TCA. The increased liberation is probably due to enzyme activity, as small amounts of inorganic phosphate were present in the extracts. Furthermore, when these extracts were treated with Buell's (1943) reagent the amount of P present in the precipitates increased with time to a maximum value after 2 days' incubation.

Dialysis was investigated as a means of separating nucleotides and nucleosides from undegraded ribonucleic acid and polynucleotide material following incubation of the pH 9-insoluble samples. Portions were suspended in water, adjusted to pH 7 and incubated at 37° for 24–36 hr.,

Table 5. *Liberation of phosphorus from pH 9-insoluble residues by further solvent action*

(Sap (1 l., 273 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (41 mg. P) was split into two equal portions, one of which was extracted with lipid solvents, before extraction at pH 9. The residues insoluble at pH 9 contained 2.5 mg. and 2.36 mg. P respectively. They were suspended in water and divided into two equal portions, and washed with water alone or with 5% (w/v) TCA followed by water. The amount of P extracted by these various treatments is recorded in the table as the % of the initial total of the precipitates. In the case of the lipid-free samples, these percentages have been calculated to include the phospholipid P removed from the sap-TCA precipitate before alkaline extraction.)

Previous treatment of sap-TCA precipitate	None	Extracted with lipid solvents
Washed with water alone		
Extract:		
Lipid solvent	—	5.7
1st water	49.2	44.2
2nd water	7.2	11.2
3rd water	3.0	4.8
4th water	0.8	2.1
Total removed by water	60.2	62.8
Total P extracted	60.2	68.5
Washed with water and TCA		
Extract:		
Lipid solvent	—	5.7
1st water	49.5	41.7
5% TCA	18.7	12.5
2nd water	5.1	11.2
3rd water	4.0	11.0
Total removed by water and TCA	77.3	76.4
Total P extracted	77.3	82.1

Table 6. *Effect of incubation of pH 9-insoluble residues on liberation of dialysable phosphorus*

(Sap from grass (mixed *Festuca* spp.) (1 l., 619 mg. P) was precipitated with TCA. The washed sap-TCA precipitate (99.7 mg. P) was treated with lipid solvents and then extracted twice at pH 9. The residue (14.7 mg. P) was suspended in water and divided into two equal portions. One was adjusted to pH 7 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The other portion was taken to pH 9 and dialysed without previous incubation. The inorganic and total P contents of the dialysates are recorded as % of the total initial P of the residue.)

	Fractions as % of total initial P	
	Incubated at 37°	Not incubated
Inorganic P	32.4	13.0
Total P	51.6	38.3

taken to pH 9 and dialysed at room temperature against NaOH, pH 9 (1.5 vol., changed every 12 hr.), till the dialysates contained less than 1–2 μg . P/ml. Control samples were brought to pH 9 immediately and dialysed in the same way. In each case, the dialysates were examined for total and inorganic P content. Incubated samples always showed marked increases over the un-incubated controls, in both inorganic and total P contents of the dialysates. The figures in Table 6 refer to lipid-free samples. Preparations from lipid-containing sap-TCA precipitates showed the same thing.

The P contained in extracts from incubated material is not solely in the form of mononucleotides. This was shown by the fact that if a sample were incubated for 24 hr. and then dialysed at pH 9 until no further P passed through the membrane, about 90% of the P of the residual material (still at pH 9) was in solution. About half of it separated with the proteins when they were precipitated from the dialysed solutions by addition of $(\text{NH}_4)_2\text{SO}_4$ to 40% saturation.

The above results clearly indicate retention by the insoluble residues of small amounts of the enzyme activity of the original sap. This was confirmed by analysis of the purine and pyrimidine contents of the incubation extracts, and by examination of the sap-TCA precipitates for presence of enzyme activity.

did not liberate further P on incubation. The extracts obtained after boiling contained less P than did those after incubation, and the ratio of purines to pyrimidines becoming soluble was greater (Table 7). As in the case of the incubated samples, more adenine was obtained when the residues were treated at 100° for 30 min. with 5% (w/v) TCA, but even so, about 10% of the initial pH 9-insoluble P still remained insoluble after these treatments. The experiment whose results are presented in Table 7, was repeated a further four times with similar results.

It will be seen that there was no significant difference in either the total amount of P extracted or in the purine and pyrimidine composition of the combined extracts, whichever of these two series of extractions of pH 9-insoluble residues was followed. However, enzyme activity occurring during incubation at 37° leads to extracts containing pyrimidines, most of which are not liberated when enzymic activity is destroyed by boiling the samples.

Phosphorus associated with protein precipitates

The figures in Table 1 show that a large proportion of the P in the pH 9 extracts of the sap-TCA precipitates separated together with the proteins when these were precipitated from the extracts. As in the case of the pH 9-insoluble residues, this appears to exist in polynucleotide combination. If the precipitates were redissolved in NaOH, pH 9,

Table 7. *Purine and pyrimidine composition of the phosphorus fractions obtained from pH 9-insoluble residues by various treatments*

(Sap (1 l., 0.213 g. P) was converted to the sap-TCA precipitate (0.047 g. P). This was treated with lipid solvents and then extracted twice at pH 9. The residue (10.34 mg. P) was suspended in water and split into two equal portions. One portion was incubated at 37° for 24 hr. at pH 7. After centrifuging down, the residue was washed twice with water on the centrifuge, treated with 5% (w/v) TCA at 100° for 30 min., centrifuged down and washed with water again. The combined extracts contained 4.63 mg. P. The other portion was treated in exactly the same way except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 4.79 mg. P in the combined extracts. The purine and pyrimidine content of the extracts is given as molecules base/100 atoms P in the combined extracts.)

Extract		Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	1.6	8.1	57.9	65.2
	Water (2)	0.8	2.0	3.8	6.9
	5% (w/v) TCA	6.2	3.6	11.7	21.4
	Water	1.8	1.3	4.3	6.5
	Total	10.4	15.0	77.7	100
Boiled 5 min.	Boiled	1.4	10.7	24.8	40.0
	Water (2)	1.0	—	5.3	6.6
	5% (w/v) TCA	5.7	2.3	34.4	42.7
	Water	1.4	—	8.7	10.7
	Total	9.5	13.0	73.2	100

Nature of phosphorus fractions liberated on incubation of pH 9-insoluble residues

Incubation of lipid-free pH 9-insoluble residues led to extracts containing predominantly pyrimidines (Table 7). The proportion of adenine in the samples, however, was much greater than in the nucleotide preparations. Further amounts of adenine were liberated when the residues after incubation were heated in 5% (w/v) TCA at 100° for 30 min. About 10% of the P present before incubation remained with the insoluble residues after these treatments.

The enzyme activity of the samples was destroyed by boiling for 5 min. After such treatment the preparations

immediately after precipitation and kept at 4° for several hours before re-precipitation, only an insignificant fraction (about 5%) of this P failed to reprecipitate.

The precipitates displayed enzymic activity. Samples were incubated at 37° in water at pH 7 for 24 hr., taken to pH 9 and dialysed against NaOH, pH 9 (1.5 vol., changed every 12 hr.), at room temperature, till the dialysates contained less than 1–2 μg . P/ml. The dialysates showed marked increases in both total and inorganic P contents over those from control samples which were dialysed at pH 9 without prior incubation. The effect was shown by both lipid-containing and lipid-free samples of proteins from sap-TCA precipitates.

About 90% of the P remaining non-dialysable after incubation was soluble at pH 9. If the proteins in these extracts were thrown out by 40% saturation with $(\text{NH}_4)_2\text{SO}_4$, 50–70% of the P was co-precipitated.

Table 8. *Effect of incubation of protein precipitates upon liberation of dialysable phosphorus*

(Sap (1 l., 212 mg. P) was treated with TCA. The sap-TCA precipitate (29.6 mg. P) was split into two equal portions. One portion was fractionated at pH 9 without removal of lipids. The other was extracted with lipid solvents prior to fractionation. The protein precipitates separating from the alkaline extracts of the sap-TCA precipitates at pH 6, contained 1.04 mg. P and 1.68 mg. P respectively. Each precipitate was then split into two equal portions. One portion was, in each case, incubated at 37° in water at pH 6 for 24 hr., taken to pH 9 and dialysed as described in the text: the remaining portions were dialysed at pH 9 without previous incubation. The inorganic and total P contents of the dialysates are recorded in the table as % of the total initial P of the protein precipitates.)

	Lipids present		Lipids absent	
	Incubated	Not incubated	Incubated	Not incubated
Inorganic P	18.5	6.0	17.7	9.8
Total P	82.7	63.5	80.6	54.0

Effect of incubation of protein precipitates on nature of phosphorus fractions liberated

Extracts obtained by incubating the protein precipitates at pH 6 for 24 hr. contained an excess of purines over pyrimidines (Table 9). Further quantities of nucleotides, also containing more purines than pyrimidines, were released when the residues were heated at 100° for 30 min. in 5% (w/v) TCA. About 10% of the P initially present failed to become soluble following these treatments.

Table 9. *Purine and pyrimidine composition of the phosphorus fractions obtained from protein precipitates by various treatments*

(Sap (1 l., 96.1 mg. P) was converted to the sap-TCA precipitate (37.8 mg. P). On treatment with lipid solvents followed by fractionation, as described in the text, this yielded a protein precipitate at pH 5 containing 3.8 mg. P. This precipitate was suspended in water and split into equal portions. One portion was incubated at 37° for 24 hr. at pH 6. After centrifuging down, the residue was washed twice with water, heated in 5% (w/v) TCA at 100° for 30 min., again centrifuged down and washed twice more with water. The extracts contained a total of 0.84 mg. P. The second portion was treated in exactly the same way, except that the initial incubation was replaced by a 5 min. boiling period to destroy enzyme activity, and yielded 0.82 mg. P in the combined extracts. The purine and pyrimidine contents of the various extracts is given as molecules base/100 atoms P in the combined extracts.)

	Extract	Adenine	Guanine	Total pyrimidines	Phosphorus
		(molecules/100 atoms total P)			(atoms)
Incubated at 37°	Incubation	15.7	45.1	6.5	69.5
	Water (2)	1.2	—	—	—
	5% (w/v) TCA	4.9	18.4	—	1.3
	Water	2.1	—	3.3	27.1
	Total extracted	23.9	63.5	9.8	2.1
Boiled 5 min.					100
	Boiled	11.9	37.5	3.4	52.2
	Water (2)	1.8	—	1.7	3.4
	5% (w/v) TCA	7.4	26.7	7.4	40.9
	Water (2)	2.2	1.1	—	3.5
	Total extracted	23.3	65.3	12.5	100

Boiling a suspension of the precipitates at pH 6 for 5 min. destroyed the enzymic activity. The boiled extracts contained rather less P than did the incubation extracts, but as was found with incubated samples, purines predominated over pyrimidines in the extracts. Treatment of the residues with 5% (w/v) TCA at 100° liberated further quantities of P, and a total of about 90% of the P initially present could be extracted from the protein precipitates by these combined treatments. Table 9 shows that approximately the same total amounts of P, of purines and of pyrimidines, are liberated, whichever set of extraction conditions are employed. However, enzymic activity during incubation of the protein precipitates gives extracts containing more nitrogen bases (especially purines) than does boiling the samples, which destroys the enzymes. Fractionation of the P of the protein precipitates by the above methods gave similar results on several more samples.

Fractionation of chloroplast-TCA precipitate

Preparation and properties of mononucleotide solutions

Chloroplast-TCA precipitate was extracted once with 95% ethanol and then with ethanol-ether (7:3, v/v) until the extracts were colourless. The lipid-free residue was then put through the same alkaline fractionation procedure as was used for the preparation of nucleotides from sap-TCA precipitates.

The amounts of P obtained in various fractions in this way, are given in Table 10. These figures refer to chloroplasts obtained from the sap preparations whose P distribution on fractionation is detailed in Table 1. The figures in these two tables differ in a number of respects. Sap-TCA precipitates contain smaller amounts of P extractable by lipid solvents (5–10% of the total) than do chloroplast-TCA precipitates. Two lipid-containing chloroplast-TCA precipitates retained only about 30% of their P after two extractions at pH 9, instead of the 40–55% remaining in samples previously treated with lipid solvents. However,

even when allowance is made for the decreased extent of extraction following removal of lipids, there is still a much smaller percentage P extraction from chloroplast-TCA precipitates than from sap-TCA precipitates. Again, only a small proportion of the P in the pH 9 extracts could be isolated in mononucleotide preparations. About half of this pH 9-soluble P is precipitated, together with the proteins, from the extracts on acidification, but its nature has not been investigated further.

Table 10. *Distribution of phosphorus in some fractions from chloroplasts*

(The values given are the extreme ranges obtained with several different batches of chloroplasts.)

	Percentage of total P of the		
	Chloroplasts	Chloroplast-TCA precipitate	pH 9 extracts
Soluble in lipid solvents	15-20	20-30	—
pH 9-insoluble residues	25-40	40-55	—
Mononucleotide preparations	2-8	4-10	15-30
Protein precipitates	10-15	15-20	45-55

Nucleotide preparations and protein precipitates account in most cases for about 70% of the alkali-soluble P. About half of the remainder is found in the aqueous ethanolic supernatants from the nucleotide precipitates. The nature of this baryta-soluble, ethanol-soluble P has not been investigated. Small amounts of P are also found associated with the BaSO₄ precipitates, and in the precipitates separating at pH 9 before addition of ethanol. It is thus usually possible to account for over 90% of the P of the chloroplast-TCA precipitate after fractionation.

The nucleotide preparations had the same general properties as did those from the sap-TCA precipitates. Analyses of typical preparations are given in Table 11, which shows that, as in the case of sap preparations, pyrimidines predominate over purines.

Table 11. *Purine and pyrimidine composition of chloroplast nucleotide preparations*

(Results of two typical experiments are presented as molecules base/100 atoms P. The chloroplasts, containing 100 mg. P in each case, gave in exp. 1 a TCA precipitate (83.1 mg. P) and nucleotide preparation (8.03 mg. P), and in exp. 2 a TCA precipitate (71.9 mg. P) and nucleotide preparation (4.03 mg. P.)

Preparation	Adenine	Guanine	Total pyrimidines
	(molecules/100 atoms P)		
1	2.1	32.1	63.6
2	0.8	34.7	61.6

Phosphorus content of pH 9-insoluble material of chloroplast-TCA precipitate

This is a considerable fraction of the initial chloroplast-TCA precipitate total (Table 10). If these preparations were incubated at 37° for 24 hr. in water at pH 6, 30-40% of the P was extracted. Repeated incubations at pH 6, the water being replaced every 24 hr., eventually led to extraction of about 70% of the P. Treatment of the residual material with 5% (w/v) TCA brought out a further 5% at room temperatures, and 15-20% after 30 min. at 100°.

Incubation of the pH 9-insoluble residues at pH 6 led to increases, over non-incubated controls, in the amount of P that could be subsequently dialysed away from the preparations at pH 9, but these increases were small.

Incubation of the pH 9-insoluble residues at 37° for 24 hr. with a sap ribonuclease preparation of the type described by Pirie (1950) (used in preference to that described in the next section because of its low dry matter and P content) in amounts more than sufficient to cause

practically complete fission in 24 hr. of a yeast nucleic acid solution (pH 6) with the same total P content as the chloroplast residues, brought out only some 50-60% of the P. Most of the remainder was released when incubation was repeated twice more with fresh enzyme solution. The remaining P (approx. 8% of the initial total) was not brought out by further incubations, or by cold or hot 5% (w/v) TCA treatment.

Table 12. *Effect of incubation upon liberation of dialysable phosphorus from residues of chloroplast-TCA precipitate insoluble at pH 9*

(Washed chloroplasts were suspended in water and precipitated with TCA. The precipitate was treated several times with lipid solvents and then extracted twice at pH 9. The residue was suspended in water and split into two equal portions. One portion was adjusted to pH 6 and incubated at 37° for 36 hr., taken to pH 9 and dialysed against NaOH, pH 9, as described in the text. The remaining portion was dialysed at pH 9 without previous incubation. In exp. 1, chloroplasts (100 mg. P) gave a TCA precipitate (52.09 mg. P) and nucleotide preparation containing 2.79 mg. P. In exp. 2, the chloroplasts (100 mg. P) gave 2.78 mg. P in nucleotide preparation from a TCA precipitate containing 56.89 mg. P. The inorganic and total P contents of the dialysates are recorded as the percentage of the total initial P of the residues.)

Experiment ...	Incubated		Not incubated	
	1	2	1	2
Inorganic P	2.1	3.9	—	—
Total P	11.6	13.2	6.1	3.0

Very little P was extracted by overnight treatment of the pH 9-insoluble residues with N-HClO₄ at room temperatures, but incubation at 37° for 24 hr. followed by washing with water brought out over 80% of the phosphorus.

The P in extracts obtained from the pH 9-insoluble residues following incubation in water (with and without added ribonuclease), or in N-HClO₄, exhibited strong ab-

sorption in the region of 260 μ . Paper chromatography of samples after hydrolysis in N-HCl for 60 min. showed the presence of adenine, guanine, cytidylic and uridylic acids. No figures have been obtained for the relative proportions of the various nitrogen bases in these extracts.

Effect of TCA precipitation upon activity of sap ribonuclease and phosphatase

Sap was split into equal halves. One lot was dialysed against distilled water at 4°, with frequent changes, for 3 days, and the volume noted. The other was precipitated with TCA and the sap-TCA precipitate, after a preliminary water wash, was suspended in water and dialysed in the same way. It was then suspended in water and made up to a known volume.

Both sap and sap-TCA precipitate preparations exhibited ribonuclease activity, with optimum fission occurring at about pH 5.5. The activity of the sap-TCA precipitate preparations was much lower than that of the sap. For example, 30% fission of a yeast nucleic acid solution was brought about by 0.125 ml. dialysed sap (equivalent to 0.107 ml. sap before dialysis) in 2.75 hr. It needed 1.5 ml. of the corresponding sap-TCA precipitate preparation (from 1.8 ml. sap before precipitation and dialysis) to give this same fission in 4.5 hr. Hence the sap-TCA precipitates still exhibited about 4% of the activity of an equivalent volume of sap.

Dialysed sap exhibited phosphatase activity towards a number of substrates. This diminished on precipitation with TCA to a much greater extent than did the ribonuclease activity. Thus, while a sample of 0.05 ml. dialysed sap (equivalent to 0.04 ml. sap before dialysis) split sodium β -glycerophosphate solution to the extent of 21% in 2 hr. at 37°, 1.5 ml. of the corresponding sap-TCA precipitate (equivalent to 1.2 ml. original sap) only caused 3% fission in 72 hr. at 37°. Hence only about 1 part in 7500 of the original phosphatase activity remained after TCA precipitation. The use of metaphosphate and yeast adenylic acid as substrates gave similar results.

DISCUSSION

Kaufmann, Gay & McDonald (1951) have shown that pancreatic ribonuclease attacks pancreatic nucleoprotein as well as the free nucleic acid. The enzyme is stable to extremes of pH, and displays marked activity at pH 9.5 (Dubos & Thompson, 1938), although it is largely inactivated in 5 min. at pH 7 and 100° (Kunitz, 1940). It would seem from the present results that the reactions leading to the formation of nucleotides from sap-TCA precipitates are at least partially enzymic in character. The results are compatible with the theory that the ribonucleoprotein in the sap-TCA precipitate is being degraded to varying extents by the sap ribonuclease. The preferential fission of pyrimidine-containing fragments from yeast nucleic acid by means of pancreatic ribonuclease has been noted by a number of workers, and has also been demonstrated for the ribonucleic acids of tobacco mosaic and turnip yellow viruses and of rabbit liver

(Markham & Smith, 1951). Carter & Cohn (1950) showed that the acid-soluble digestion products produced from yeast nucleic acid by ribonuclease consisted almost exclusively of pyrimidine nucleotides, and suggested that the very small amounts of adenylic and guanylic acids also isolated were artifacts obtained during the isolation procedure from material of higher molecular weight.

The presence of purines in the nucleotide mixtures obtained here from sap may be due to purely chemical degradation of the nucleic acid (cf. Schmidt & Thannhauser, 1945). However, Pirie (1950) has already noted that sap ribonuclease differs from the pancreatic enzyme in that it brings about more or less complete fission of yeast and sap ribonucleic acids, so that the products are no longer capable of precipitation by the uranyl reagent. Moreover, Kunitz (1940) reported an optimum pH of 7.7 for pancreatic ribonuclease activity, whereas the sap enzyme has been found to exhibit maximum activity at pH 5-6. It is, therefore, possible that the presence of purines in the sap preparations is due to ribonuclease activity, and this is supported by the exhibition of enzymic activity, leading to the almost exclusive liberation of purine nucleotides, by the protein precipitates obtained during isolation of the nucleotide preparations. The low yield of nucleotides obtained when pH 9-insoluble material and protein precipitates were pooled and again fractionated also agrees with enzymic, as opposed to purely chemical, decomposition during alkaline fractionation.

Bacher & Allen (1950) recently showed that the usual methods of preparation of pancreatic ribonucleic acid yielded products containing ribonuclease. Pirie (1950) described the similar association of ribonuclease activity with tobacco sap nucleoprotein. He also showed the partial breakdown of the sap nucleic acid when prepared from the nucleoprotein by decomposition with TCA, even fresh preparations yielding a maximum of only 60% of the phosphorus in the nucleic acid. Starting with sap, he was unable to obtain any undegraded nucleic acid by precipitation with TCA, and he ascribed this partly to decomposition on prolonged contact of the nucleoprotein with TCA, and partly to enzyme action in the sap or during the preparation. The present results are in agreement with these conclusions. Enzyme activity in the sap-TCA precipitate and in various fractions obtained during the fractionation procedure, has been amply demonstrated. The evidence concerning the effect of contact with TCA on the sap-TCA precipitate, although it is not unequivocal, as the results were somewhat variable, also supports the above conclusions. Most of the present work has been done on sap-TCA precipitates collected immediately after preparation, but a small number of experiments

were performed in which these precipitates were allowed to age in contact with the TCA supernatant before being collected. In general, rather more phosphorus was found in a precipitate collected immediately after preparation than in an aged precipitate, the difference being greater the longer the precipitate was aged. It appears that sap nucleic acid is more sensitive to decomposition by TCA than are other nucleic acids (cf. Schneider, 1945). Ageing a sap-TCA precipitate decreased the solubility of the sap proteins at pH 9, but the part that went into solution retained on reprecipitation an amount of phosphorus larger than that separating with the proteins from a fresh sap-TCA precipitate. This is probably due to preferential absorption of nucleic acid or polynucleotide material by the denatured proteins.

The formation of nucleotides from chloroplast-TCA precipitates probably follows the same pattern as for sap. However, the smaller proportion of material soluble at pH 9, and of nucleotides isolable from this material, suggests either that the ribonucleic acid of chloroplasts is present in two fractions, one of which is not readily accessible to ribonuclease action, or that chloroplasts contain a ribonuclease inhibitor. Repeated additions of large amounts of sap ribonuclease were found to be necessary to extract the remaining nucleic acid from chloroplast residues, so that the low extent of extraction from the initial TCA precipitate and the slow rate of liberation of phosphorus from the pH 9-insoluble residues, do not appear to be due to enzyme inhibition. Moreover, it has recently been found (Holden, 1951) that only about 40% of the chloroplast ribonucleic acid is extracted by $N\text{-HClO}_4$ at room temperature, incubation at 37° being necessary to remove the remainder. Ogur & Rosen (1950) claimed complete extraction of ribonucleic acid from plant tissues under the former conditions.

The existence of phosphatase activity in the pH 9-

insoluble residues and the protein precipitates is rather surprising in view of the low activities found for the sap-TCA precipitates. It is possible that traces of the enzyme escape denaturation during TCA precipitation, through being carried down mechanically in the precipitate, and are subsequently released when the precipitates are brought to pH 9.

It was noticed during incubation of pH 9-insoluble residues, suspensions of protein precipitates and of sap-TCA precipitates, that the suspensions rapidly settled and changed gradually, over a period of several hours, to a harder, more compact form. This did not affect the enzymic activity of the samples, since agitation did not enhance it, although it delayed completion of these changes.

SUMMARY

1. A fractionation procedure for following the breakdown of sap and chloroplast ribonucleic acids at pH 9, following precipitation with trichloroacetic acid, is described.

2. Both sap and chloroplast nucleic acids were partially degraded to a mixture of mononucleotides containing an excess of pyrimidines over purines.

3. The remaining breakdown products were found to be contained, in polynucleotide combination, principally in material insoluble at pH 9 and in protein precipitates obtained during fractionation. The purine and pyrimidine distribution following enzymic and chemical degradation of these fractions is described.

4. Differences in the ease of degradation of sap and chloroplast nucleic acids are described.

5. The extent of denaturation of sap ribonuclease and phosphatase following precipitation with trichloroacetic acid was determined.

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Amine Oxidase Activity of Rat Liver in Riboflavin Deficiency

By JOYCE HAWKINS

*Department of Pharmacology, Oxford**

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The chemical constitution of the enzyme amine oxidase is not known. There are some indications that the closely related enzyme histaminase, which also catalyses the oxidation of amines, is a flavo-protein (Zeller, Stern & Wenk, 1940; Kapeller-Adler, 1949). It is known that D-amino-acid oxidase contains riboflavin in the form of flavin-adenine dinucleotide (FAD) (Warburg & Christian, 1938). In riboflavin deficiency there occurs a marked reduction in the D-amino-acid oxidase activity of rat liver (Axelrod, Soker & Elvehjem, 1939, 1940; Rossiter, 1940), and the FAD content of the liver is reduced (Ochoa & Rossiter, 1939).

In the present work the effect of riboflavin deficiency on the amine oxidase activity of rat liver has been studied. The D-amino-acid oxidase activity has been determined at the same time in order that this might serve as an index of the degree of the riboflavin deficiency obtained. In this study characteristic differences in the two enzymes have been observed.

METHODS

Diet. The method adopted for inducing riboflavin deficiency in rats was based on that described by Carpenter Harris & Kodicek (1948) who used a diet low in protein and containing succinylsulphathiazole. I am grateful to Dr E. Kodicek for his advice on the use of this diet.

The diet was composed as follows: riboflavin-free casein 10.5%, sucrose 80.35%, salt mixture (Steenbock no. 40) 5%, L-cystine 0.15%, arachis oil 3%, and succinylsulphathiazole 1%. This diet was fed to the rats *ad lib.* As supplements, they received daily: 30 µg. aneurin, 30 µg. pyridoxine, 20 mg. choline chloride, 1 µg. biotin, 250 µg. nicotinic acid and 200 µg. calcium pantothenate; three times weekly they were given 40 µg. folic acid dissolved in 1% KH_2PO_4 , and once a week one drop of a 5% solution of toco-

pherol acetate, four drops of cod-liver oil and 1 mg. menaphthone. The animals which received this diet without added riboflavin are called deficient rats. The animals which received a daily supplement of 30 µg. riboflavin are called controls. Modifications of the diet, as used in some of the experiments, will be described later.

Animals. Litters of freshly weaned male and female piebald rats, obtained from Glaxo Laboratories, were used. They weighed about 30 g. on arrival.

About two-thirds of the rats in each litter received the deficient diet. In most litters the remaining animals were kept as controls and received daily supplements of riboflavin.

Tissue preparations. Each liver was homogenized in a small volume of 0.067M-sodium phosphate buffer, pH 7.4. More buffer was added so that 5.0 ml. homogenate contained 1 g. tissue. About one-half of the homogenate was retained for amine oxidase determination, the remainder was centrifuged at 600g for 5 min. and the supernatant used for D-amino-acid oxidase determination.

Kidney extracts were prepared by first grinding the tissue with sand in a mortar, and then adding 0.067M-sodium phosphate buffer, pH 7.4, so that 5.0 ml. suspension contained 1 g. tissue. This suspension was centrifuged for 5 min. at 600g and the supernatant was used for D-amino-acid oxidase determination.

Measurement of enzymic activity. All determinations were made at 37° and in O_2 . Conical manometer flasks were used; the inner compartments contained filter paper and 0.3 ml. N-KOH.

Amine oxidase activity. This was determined with tyramine as substrate. The side bulbs contained either 0.4 ml. of 0.05M-tyramine hydrochloride, or 0.4 ml. of water in the blank. The main compartment of each flask contained 1.0 ml. of rat-liver homogenate plus 0.2 ml. of 0.1M-semicarbazide plus 0.4 ml. of water, to trap the aldehyde formed during the initial oxidation of tyramine in order to prevent further oxidation.

For the study of D-amino-acid oxidase, DL-proline was chosen, as D-proline was found to be oxidized at a high rate by the rat enzyme. The side bulb contained 0.5 ml. 1.0M-DL-proline; the main compartment contained 0.5 ml. liver extract plus 0.5 ml. water. In the experiments with kidney the proline concentration was 0.1M.

* Present address: Department of Chemistry and Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1.

Readings were taken at 5 min. intervals, and the rate of oxidation was calculated from the O_2 uptake during the first 15 min. of the reaction. Enzymic activity is expressed in terms of q_{O_2} (tyramine) and q_{O_2} (proline) respectively, i.e. in μ l. of O_2 /100 mg. fresh wt. tissue/hr.

EXPERIMENTS

Riboflavin deficiency and enzymic activity of liver preparations

Rats reared on the control diet showed a slow but steady growth and appeared healthy; they almost doubled their initial body weight after a fortnight. The deficient animals showed very little or no growth. Apart from diarrhoea, which became noticeable in almost all deficient animals, there were no symptoms of deficiency. The animals on the deficient diet usually survived for a fortnight, but during the third week some deaths occurred. One set of animals was maintained for a fortnight (13–15 days), and a second for 3 weeks (20–22 days). All rats in one litter were killed at one time and the enzymic activities were determined.

Administration of riboflavin and enzymic activity

It is known that on administration of riboflavin to flavin-deficient rats the D-amino-acid oxidase activity of the liver is rapidly restored (Axelrod *et al.* 1940). I have compared the effect on amine oxidase of adding riboflavin to the riboflavin-deficient diet with the effect on D-amino-acid oxidase.

In these experiments about one-third of the animals in each litter were kept as controls, one-third were maintained on the deficient diet throughout, and the remaining third were kept on the deficient diet for 11 or 12 days and then given 1 mg. riboflavin by mouth. In one series the animals received one such dose of riboflavin and were killed 24 hr. later. In a second series the rats were given 1 mg. doses of riboflavin on three successive days, and killed 24 hr. later.

The effect of riboflavin on the growth curve was immediate; the mean gain in wt. after 1 mg. of riboflavin was 4 g., and after three doses of 1 mg. riboflavin it was 7 g. The enzymic activities of the

Table 1. *Liver amine oxidase and D-amino-acid oxidase from control and riboflavin-deficient rats*

(Experimental details are given in the Methods section. In this and subsequent tables values are shown as means \pm S.E. with the number of observations in brackets.)

Time on diet (weeks)	Diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase
2	Control	115 \pm 8 (23)	124.5 \pm 11.5 (18)
2	Deficient	61.5 \pm 3.5 (24)	24.5 \pm 1.5 (24)
3	Control	122 \pm 8 (11)	142 \pm 6.5 (11)
3	Deficient	54.5 \pm 5.5 (14)	18 \pm 3 (14)

Table 2. *The effect of riboflavin administration on the enzymic activity of liver extracts from deficient animals*

(Experimental details are given in Methods section.)

Diet	Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase
Control	None	164 \pm 7.1 (9)	136 \pm 12.8 (9)
Deficient	None	62.5 \pm 3.9 (10)	25 \pm 4.4 (10)
Deficient	One dose 1 mg. riboflavin	48 \pm 7.5 (4)	70 \pm 21 (4)
Deficient	Three daily doses 1 mg. riboflavin	64.5 \pm 2.5 (5)	170 \pm 15 (6)

The enzymic activities of the liver from control and deficient animals are given in Table 1. After 2 weeks on the experimental diet the D-amino-acid oxidase activity in the liver extracts from deficient animals had fallen to one-fifth of that of the controls. The amine oxidase activity was reduced to 53 %. When the animals were maintained on the diet for a third week the D-amino-acid oxidase activity fell to 13 % and the amine oxidase activity to 45 % of the controls.

Changes in the total amount of enzyme present in the liver will be described below.

liver after riboflavin administration are shown in Table 2. It can be seen that the D-amino-acid oxidase activity was increased by one dose of riboflavin, whereas the amine oxidase activity remained essentially unchanged even after three doses of riboflavin. After three doses of riboflavin the D-amino-acid oxidase activity rose to a level higher than that of the controls.

The determination of the enzymic activity per unit weight of liver does not give a full picture of the effect of riboflavin administration on the restoration of the enzymes studied, because administration of

riboflavin causes a rapid increase in liver weight. The body and the liver weights of rats on the different diets are summarized in Table 3. The mean liver weight rose from 1.66 to 2.60 g. after one dose of riboflavin, and to 3.10 g. after three doses. The total enzymic activity can be measured in $\mu\text{l. O}_2$ consumed/hr./liver; this is the q_{O_2} multiplied by the liver weight (in units of 100 mg.). The mean data thus obtained are given in Table 4 which shows that after administration of riboflavin the total amine oxidase of the liver increased. However, this increase appears slight if it is compared with the big rise in total D-amino-acid oxidase activity. Thus, after three daily doses of 1 mg. of riboflavin, amine oxidase had about doubled, whereas D-amino-acid oxidase had increased about 13-fold in these animals.

Other observers have reported that in rats on a fat-free diet low in protein and containing choline, the administration of large amounts of B vitamins resulted in the deposition of fat; this was partly prevented by inositol (McFarland & McHenry, 1948; Best, Lucas, Patterson & Ridout, 1951). Since the basic diet used in my experiments was low in protein and fat and did not include inositol, it was thought desirable to study the effect of inositol on the restoration of enzymic activity in the liver after the administration of riboflavin.

In the first series of experiments inositol was not included in the basic diet. Two litters were used; in one litter three of the deficient animals received three daily doses of 25 mg. inositol on the last 3 days of the experiment. In the second litter three animals received three daily doses of 25 mg.

Table 3. *Body weights and liver weights of rats maintained on deficient and control diets*

Diet	Time on diet (weeks)	Supplement to diet	Mean body wt. (g.)	Liver wt. (g.)	No. of obs.
Control	3	None	66	3.75 \pm 0.08	(12)
Deficient	3	None	42	1.83 \pm 0.11	(13)
Control	2	None	55	3.08 \pm 0.11	(21)
Deficient	2	None	34	1.66 \pm 0.14	(20)
Deficient	2	One dose 1 mg. riboflavin	38	2.60 \pm 0.05	(4)
Deficient	2	Three daily doses 1 mg. riboflavin	41	3.10 \pm 0.15	(6)
Control + inositol	2	None	47	2.85 \pm 0.19	(6)
Deficient + inositol	2	None	35	1.64 \pm 0.08	(11)
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	45	2.68 \pm 0.13	(10)

Table 4. *Total amine oxidase and D-amino-acid oxidase activities of the liver, expressed in $\mu\text{l. O}_2$ /hr./liver*

Diet	Time on diet (weeks)	Supplement to diet	Amine oxidase ($\mu\text{l. O}_2$ /hr.)	D-Amino-acid oxidase ($\mu\text{l. O}_2$ /hr.)
Control	3	None	4560	5350
Deficient	3	None	996	256
Control	2	None	3540	3840
Deficient	2	None	1020	405
Deficient	2	One dose 1 mg. riboflavin	1250	1820
Deficient	2	Three daily doses 1 mg. riboflavin	2000	5270
Control + inositol	2	None	3420	3690
Deficient + inositol	2	None	1130	312
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	2800	5070

Inositol and the restoration of enzymic activity by riboflavin

In the course of the experiments just described, it was noticed that after administration of riboflavin, the livers of deficient rats showed an increase in fat content. This became particularly evident after centrifugation of the liver extracts, when a well-defined layer of fat formed on the surface of the extracts in the centrifuge tubes. This suggested that the rapid increase in liver weight after riboflavin administration might, at least partly, be explained by an increase of the fat content of the liver.

inositol together with 1 mg. of riboflavin. The animals were killed 24 hr. after the last dose and the enzymic activities were determined. Table 5 gives a summary of the results obtained with these litters, and for comparison, the effect of three daily doses of riboflavin alone is also given. The mean enzymic activities of the deficient animals are those from both litters; the values differ little from the mean values given in Table 1. Inositol by itself had no significant effect on either amine oxidase or D-amino-acid oxidase activity, neither did it affect the restoration of D-amino-acid oxidase after riboflavin. The combined administration of riboflavin and

inositol, however, resulted in an increase in the amine oxidase activity of the liver extracts.

In the second series of experiments inositol was included in the basic diet. Table 6 shows that both amine oxidase and D-amino-acid oxidase activities from normal and deficient rats were very much the same as in the absence of inositol. However, when three daily doses of 1 mg. of riboflavin were given to the deficient animals there occurred a rise in the q_{O_2} (tyramine) from 69 to 104.5. In the absence of inositol, the corresponding figures had been 61.5 in the deficient animals and 64.5 after the administra-

D-Amino-acid oxidase activity of the kidney

In a number of experiments the D-amino-acid oxidase activity of the kidney was also determined. The mean enzymic activities found in these experiments are given in Table 7. The activity was high in the kidney extracts from control animals, and there was no significant lowering in the deficient animals. This is in agreement with the observations of Axelrod *et al.* (1940). It seems doubtful also whether the slight increases in q_{O_2} (proline) after three doses of riboflavin were significant.

Table 5. *The effect of inositol and riboflavin on the enzymic activity of liver from animals maintained on a riboflavin-deficient diet*

(Experimental details are given in the Methods section.)

Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
None	63.5 \pm 2.2 (5)	30 \pm 3.4 (5)
Three daily doses 25 mg. inositol	69 \pm 8.5 (3)	37 \pm 10.5 (3)
Three daily doses 1 mg. riboflavin + 25 mg. inositol	88.5 \pm 3.5 (3)	163 \pm 24 (3)
Three daily doses 1 mg. riboflavin	64.5 \pm 2.5 (5)	170 \pm 15 (6)

Table 6. *Enzymic activities of liver extract from animals on a basic diet plus inositol*

(Each rat received 8 mg. inositol/day. The experiment lasted 14 days. The corresponding mean enzyme activity of liver from rats on the basic diet alone is given in square brackets.)

Diet	Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
Control	None	120 \pm 9.5 [115] (5)	129.5 \pm 19.5 [124.5] (6)
Deficient	None	69 \pm 6.5 [61.5] (11)	19 \pm 2 [24.5] (10)
Deficient	Three daily doses 1 mg. riboflavin	104.5 \pm 6.5 [64.5] (10)	189 \pm 16 [170] (10)

Table 7. *D-Amino-acid oxidase of rat kidney*

(Experimental details are given in the Methods section.)

Diet	Time on diet (weeks)	Supplement to diet	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
Control	3	None	251 \pm 27 (5)
Deficient	3	None	243 \pm 12 (8)
Control	2	None	255.5 \pm 19.5 (11)
Deficient	2	None	237 \pm 18.5 (15)
Deficient	2	One dose 1 mg. riboflavin	230 \pm 46.5 (4)
Deficient	2	Three daily doses 1 mg. riboflavin	304.5 \pm 26 (6)
Deficient	2	One dose 1 mg. riboflavin + 25 mg. inositol	203 \pm 31.5 (3)
Deficient	2	Three daily doses 1 mg. riboflavin + 25 mg. inositol	297 \pm 61 (3)
Deficient	2	Three daily doses 25 mg. inositol	236 \pm 28 (3)

tion of riboflavin. No effect of inositol on the restoration of D-amino-acid oxidase activity was seen.

The effect of riboflavin on the total enzymic activities of the liver of rats maintained on the inositol-containing diet is shown in Table 4. On this diet, the D-amino-acid oxidase activity rose from 312 to 5070 μ l. O_2 /hr., about 16-fold; the amine oxidase activity rose from 1130 to 2800 μ l. O_2 /hr., about 2.5-fold.

Enzymic activity on a high-protein diet

In order to find out if the lowered amine oxidase activity was due to a low protein intake, a few animals were reared on a diet which differed from the basic diet in that the casein content was raised from 10 to 20 %, with a corresponding reduction in the sucrose to 70.35 %. On this diet the control animals grew more rapidly than on the low-protein diet: the weights of three control animals kept for

3 weeks on the high-protein diet were 86, 89 and 89 g. as compared with a mean weight of 66 g. after 3 weeks on the low-protein diet (see Table 3). The weight of the riboflavin-deficient rats on the high-protein diet did not remain stationary; the rats put on weight during the first 2 weeks; some of the animals were maintained for as long as 5 weeks without showing any symptoms.

Three of the deficient animals were killed after 21 days and liver extracts were prepared; the mean q_{O_2} (tyramine) was 78 and the mean q_{O_2} (proline) was 19. Eight deficient and three control animals were killed after 30–38 days. Table 8 shows that the fall in both amine oxidase and D-amino-acid oxidase activities had not been prevented by increasing the protein content of the diet; the mean values for q_{O_2} are similar to those of Table 1.

control animals. The data given by Axelrod *et al.* (1940), although not strictly comparable with those reported in this paper, suggest that on the diets used by them the decrease in enzymic activity was less rapid. Thus it seems that the inclusion of succinylsulphathiazole in the diet reduced the time required for establishing a riboflavin deficiency, as indicated by the fall in D-amino-acid oxidase activity. That this fall was due specifically to lack of riboflavin and not to low protein content of the basic diet, is supported by the observation that a similar fall in enzymic activity occurred when the protein content of the diet was doubled.

D-Amino-acid oxidase of the kidneys. The maintenance of both FAD content and D-amino-acid oxidase activity of the kidneys in riboflavin deficiency has already been described (Rossiter,

Table 8. *Enzymic activity of liver extracts from animals maintained for 30–38 days on a diet containing 20% protein*

(Experimental details are given in the Methods section.)

Diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)	Total amine oxidase (μ l. O_2 /hr./liver)	Total D-amino- acid oxidase (μ l. O_2 /hr./liver)
Control	132 \pm 13.4 (3)	64 \pm 4.1 (3)	6700	3270
Deficient	50 \pm 3.5 (8)	19 \pm 4.4 (8)	1260	466
Deficient, then 3 daily doses of 1 mg. riboflavin	86 \pm 12 (5)	134 \pm 13.6 (5)	3948	5895

Some of the animals were maintained on the high protein diet without riboflavin for 5 weeks; they then received three daily doses of 1 mg. of riboflavin before they were killed. The data for enzymic activity of the liver are also given in Table 8. As in the earlier experiments, D-amino-acid oxidase activity was restored; there was, however, also some increase in the amine oxidase activity: the mean figures for q_{O_2} (tyramine) rose from 50 to 86. Table 8 also contains the data for the total enzymic activity of the liver; these show that the amine oxidase had trebled after three doses of riboflavin. The mean liver weight of the deficient animals was 2.51 g.; it increased to 4.56 g. after riboflavin. The mean liver weight of the control animals was 5.12 g.

DISCUSSION

The experiments reported confirm that on a diet free from riboflavin which incorporates succinylsulphathiazole, rats quickly develop a state of deficiency; the rapid increase of body weight which occurs when riboflavin is added to the diet is evidence of the specific character of this condition.

D-Amino-acid oxidase of liver. After about 2 weeks on the riboflavin-deficient diet the enzymic activity dropped to about one-fifth of that of the

1940; Axelrod *et al.* 1940); in my experiments also no fall in enzymic activity could be found. The riboflavin present as FAD in the D-amino-acid oxidase of the kidneys represents such a small fraction of the total flavin content of the body that a great reduction in flavin can occur while the enzymic activity of the kidneys is maintained.

Amine oxidase. The observations on this enzyme stand in marked contrast to those on D-amino-acid oxidase. The loss of amine oxidase in the flavin-deficient animal was slow, as was the rate of increase in activity after riboflavin administration. This makes it impossible to decide whether or not this enzyme contains flavin. Loss of enzymic activity occurred on the high-protein diet as well as on the low-protein diet. The possibility remains, however, that in riboflavin deficiency protein utilization is affected and that this factor is responsible for the low amine oxidase activity in the deficient animals. It seems particularly interesting that with inositol present in the diet, riboflavin was more effective in restoring amine oxidase activity. This may mean that both flavin and inositol are contained in enzyme protein, but it is also possible that amine oxidase is a flavoprotein, and that inositol is required, either for the synthesis of a prosthetic group or of new enzyme protein.

SUMMARY

1. Rats have been made deficient in riboflavin on a diet free from riboflavin and containing 1% succinylsulphathiazole and the amine oxidase and D-amino-acid oxidase of liver extracts have been determined.

2. It has been confirmed that the D-amino-acid oxidase of the liver is rapidly and severely reduced

in riboflavin deficiency and that it is rapidly and fully restored upon the administration of riboflavin.

3. The amine oxidase activity of the liver is about halved in riboflavin deficiency. There is no rapid restoration of enzymic activity on giving riboflavin. If inositol is present in the diet, some restoration of amine oxidase occurs when riboflavin is administered.

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The Effect of Thyroxine and Thiouracil on the Composition of Milk

2. THE CAROTENE AND VITAMIN A CONTENT

BY R. CHANDA AND E. C. OWEN

The Hannah Dairy Research Institute, Kirkhill, Ayr

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The interactions between the utilization of carotene as a provitamin A and the activity of the thyroid gland have been much studied both for animals and for man (Kunde, 1926; Lindquist, 1938; Thomson *et al.* 1939; Drill, 1943). Experiments with rats provide a basis for the connexion (long known to clinicians and quoted by Lindquist and by Drill) between carotene and the thyroid in certain types of human illnesses. Thus it has been shown in rats (Johnson & Baumann, 1947) that thyroxine accelerates repletion of hepatic stores of vitamin A from dietary carotene, whilst thiourea has the reverse effect. Thyroxine also accelerates the depletion of hepatic stores of rats deprived of dietary carotene whilst thiourea again has the reverse effect (Kelley & Day, 1948). Furthermore, Cama & Goodwin (1949) have correlated these effects with those produced by the hormone and by a goitrogen on the faecal excretion of dietary carotene in the rat.

For many years effects of thyroxine injections on the composition of milk have been studied at this Institute (Smith & Dastur, 1940; Owen, 1948; Chanda & Owen, 1949) and it was thought that the influence of thyroxine on the liver reserves of vitamin A of animals and humans might have a parallel in its effects on the concentration of vitamin

A and carotene in milk. Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) claimed that thyroidectomy caused carotene to appear in goat's milk, but Kon and his co-workers (1944-6) and Smith, Niedermeier & Schultz (1948) could not confirm these observations. The purpose of the present paper is to report experiments which were begun in 1948 on lactating cows and goats in which effects of thyroxine and of the goitrogen thiouracil on the composition of milk were investigated.

EXPERIMENTAL

Experiment 1

This experiment was divided into seven periods. Six lactating Ayrshire cows were used. In period 1 all were deprived of carotene and in period 2 they all received a carotene-containing diet. In period 3 all the cows were again deprived of carotene. In period 4, the diet containing carotene was fed again to all, but cows 17 and 18 were also given subcutaneously 10 mg. DL-thyroxine/day each, while cows 19 and 20 each received 20 mg. thiouracil/day by the same route. The remaining two cows served as controls. In period 5, the carotene-containing diet was continued, but the hormonal treatments were stopped. The experiment was continued into periods 6 and 7, during which the cows all received the carotene-free diet. During period 6 each cow was given the same 'hormonal' treatment as it had received

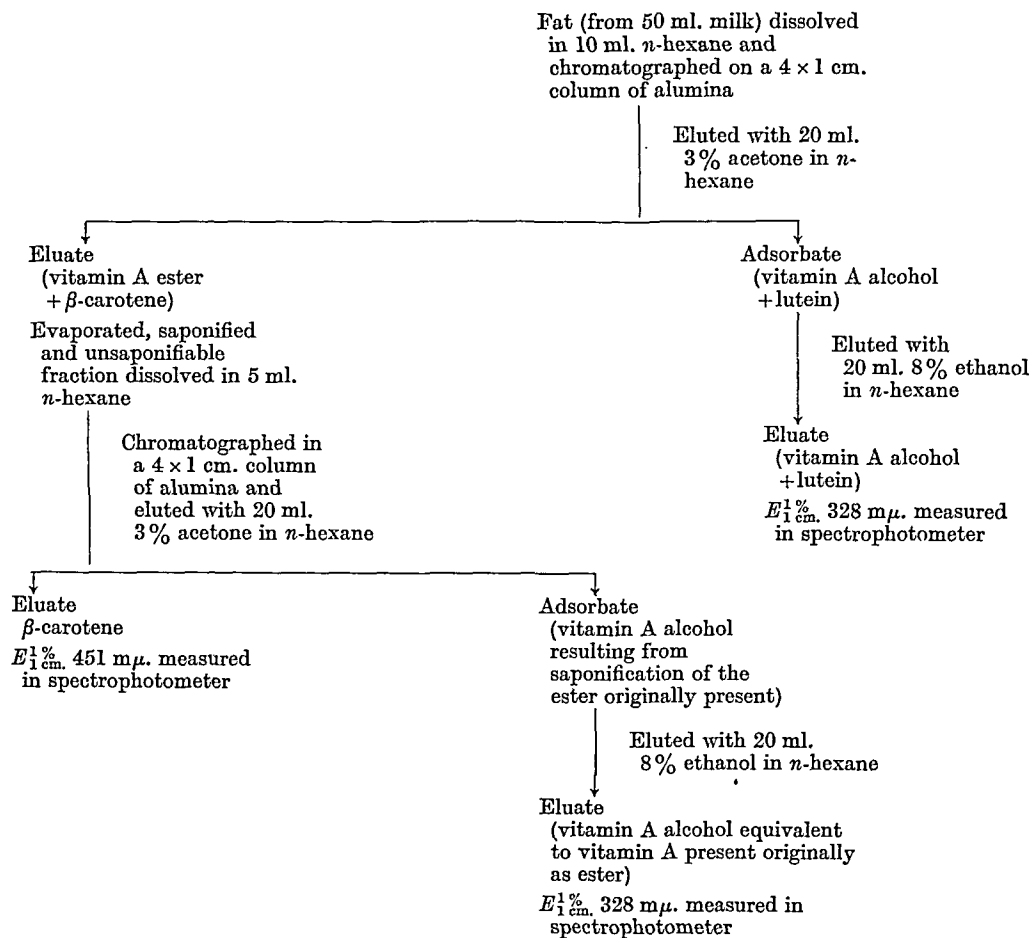
previously in period 4. In period 7 the hormonal treatments were discontinued, while all six cows were still being deprived of carotene. The duration of each period can be seen from Fig. 2.

Diets in Experiment 1. The preparation of the diets for Exp. 1 was as described by Chanda, Clapham, McNaught & Owen (1951*a, b*). In both carotene-containing and carotene-free diets the roughage was 10 lb. oat straw/cow/day fed in two equal portions, one in the morning and one in the afternoon.

The concentrate mixture of the carotene-containing diet consisted of 9 parts whole oats, 6 parts beans and 6 parts dried grass (all by weight).

Experiment 2

Eight lactating goats were used, one British Alpine (no. 5) and the rest British Saanens. The experiment was divided into three periods, and the hormonal treatments were confined to period 2. The goats received throughout a daily ration of 3 lb. of a concentrate mixture consisting of 9 parts by weight of oats, 6 parts of groundnut cake and 6 parts of dried grass. A carotene-free roughage was provided by oat glumes. Each goat received 1 lb. oat glumes per day in two equal portions. Water was given *ad lib*. The goats were divided into four pairs for treatment. During period 2 each goat of one pair received daily 10 mg. thy-



The carotene-free diet was designed to have the same content of digestible nutrients and digestible protein as the carotene-containing diet. Accordingly it contained, instead of dried grass, a mixture of whole blood, dried at low temperature (W. and J. Dunlop, Dumfries) 0.90 parts, ground straw 2.55 parts, and dry potato starch 2.55 parts (all by weight), making in all 6 parts to replace the 6 parts of dried grass in the other diet. This mixture, together with oats (9 parts) and beans (6 parts), formed the concentrate for the carotene-free rations. Each cow received 21 lb. of mixture each day in three equal portions, one in the morning, another at mid-day and the third in the evening.

roxine, each of a second pair 20 mg. thiouracil and each of a third pair 10 mg. thyroxine, together with 1 mg. stilboestrol. The remaining pair were untreated controls. Thyroxine and thiouracil were subcutaneously injected in aqueous solution. The stilboestrol was given by the same route in solution in 1 ml. olive oil (Malpress & Owen, 1947). The dose of thyroxine proved rather too large, for the goats developed symptoms of hyperthyroidism. They rapidly lost weight, developed a wrinkling of the palpebrae and their breathing became rapid but shallow. Their appetites were adversely affected, with simultaneous polyuria. Polyuria, resulting from thyroxine treatment, has been noted before

in cows by Owen (1948). The dose of thyroxine was, however, based on the experiments of Meites & Turner (1948). Possibly the humidity of the Scottish summer made it more difficult for our goats to dispose of the heat burden which thyroxine treatment necessarily causes. In continental America, where Meites & Turner worked, the average humidity was perhaps not so high.

On alternate days fat and vitamin A were estimated in goat's milk. Cow's milk from alternate evening milkings was analysed for vitamin A and carotene. The goats' milk was expressed by hand, and that of the cows was taken from the recorder vessel.

Chemical methods

Separation of carotene, lutein, vitamin A alcohol and vitamin A ester present in milk fat. Milk fat was extracted by the method of Olson, Hegstedt & Peterson (1939). The carotene, lutein, vitamin A alcohol and vitamin A ester were separated as described by Chanda, Owen & Cramond (1951). The method was based on that of Ganguly, Kon & Thompson (1947), and is summarized in the scheme shown on p. 405, which gives an outline of the chromatographic treatment of the fat.

(1933) was used to extract vitamin A and carotenoids from liver and to extract vitamin A from kidneys. These extracts were then chromatographed by the procedure used for milk.

RESULTS

Differences between the compositions of cow's and goat's milk

The reliability of the scheme of analysis outlined on p. 405 was tested by measuring the recoveries of vitamin A alcohol and ester which had been added to milk fat. Duplicate samples of milk fat were dissolved in *n*-hexane, and measured amounts of pure vitamin A were added to one of each pair of samples of fat. To some samples vitamin A was added as the alcohol, to others as the ester. The results of spectrophotometric measurements are shown in Table 1 from which it will be seen that with 30 i.u. vitamin A, as ester or as alcohol, 93 % could be recovered when added to milk fat of either the cow or the goat. Even with as little as 5 i.u., 80 % could be recovered (Table 1).

Table 1. *The recovery of different forms of vitamin A added to milk fat*

(Two lots of fat were extracted from each milk sample and taken up in *n*-hexane. Pure vitamin A in *n*-hexane was added to this solution prior to chromatography in one lot; the other was kept as a control. All spectrophotometric readings were corrected for irrelevant absorption by the method of Morton & Stubbs (1946).)

Source of milk fat	Vitamin A in original milk fat from 100 ml. milk (i.u.)		Vitamin A added (i.u.) in the form of		Vitamin A in fortified milk fat (i.u.)					
					Ester			Alcohol		
					Recovery of added vitamin (%)		100 (e-a)/c	Recovery of added vitamin (%)		100 (f-b)/d
	Ester (a)	Alcohol (b)	Ester (c)	Alcohol (d)	Expected (a+c)	Found (e)		Expected (b+d)	Found (f)	
Cow 1	71	6	50	—	121	120	98	6	5	—
2	67	4	30	—	97	95	93	4	5	—
3	99	7	—	10	99	96	—	17	15	80
4	88	5	—	20	88	92	—	25	23	90
5	77	5	—	30	77	80	—	35	34	97
6	84	4	—	50	84	81	—	54	52	96
Goat 1	184	—	30	—	214	212	93	—	—	—
2	122	—	50	—	172	170	96	—	—	—
3	106	—	—	5	106	105	—	5	4	80
4	132	—	—	10	132	129	—	10	8	80
5	115	—	—	20	115	118	—	20	18	90
6	155	—	—	30	155	159	—	30	29	97
7	165	—	—	40	165	161	—	40	38	95
8	141	—	—	50	141	138	—	50	47	94

Optical measurements were made in a Unicam spectrophotometer. Total vitamin A was also determined as vitamin A alcohol in a separate sample of fat after saponification and chromatography. All the spectrophotometric readings were corrected for irrelevant absorption by the three-point method of Morton & Stubbs (1946), as previously reported by Chanda, Owen & Cramond (1951). By this means Glover, Goodwin & Morton (1948) showed that it is possible to determine vitamin A in the presence of a preponderant irrelevant absorption.

Analysis of liver and kidney. The procedure of Davies

The method was applied to cow's and goat's milks with the results shown in Tables 2 and 3 in which milk yields and percentages of fat are also recorded. The cow's milk contained appreciable amounts of carotene (Table 2), but no carotene could be found in the goat's milk. Cow's milk contained about 6 % of its vitamin A as alcohol, but in goat's milk no measurable amount of vitamin A alcohol was present. Goat's milk contained more vitamin A per g. fat (39 i.u.) than cow's milk (21 i.u.).

Table 2. *Carotene and vitamin A in cow's milk*
(6-8 weeks post partum.)

	Cow no.						Mean	c.v.*
	1	2	3	4	5	6		
Milk yield (kg./2 days)	30.2	28.6	28.8	28.9	32.0	29.7	29.7	4.3
Fat in milk (%)	4.1	4.4	3.9	4.2	4.7	3.9	4.2	7.4
β -Carotene (μ g./100 ml.)	18.5	12.1	19.5	17.6	19.4	16.5	17.3	16.0
Total vitamin A (a) (i.u./100 ml.)	79.5	71.4	83.8	89.7	107.9	93.5	87.6	10.2
Vitamin A ester as % of (a)	89.4	93.7	91.9	93.5	92.5	94.0	92.5	1.9
Vitamin A alcohol as % of (a)	7.5	4.7	5.9	4.6	6.5	5.4	5.8	19.2
β -Carotene (μ g./g. fat)	4.5	2.8	5.0	4.2	4.1	4.2	4.1	18.2
Vitamin A (i.u./g. fat)	19.4	16.2	21.5	21.4	23.0	24.0	20.9	13.3

* c.v. (coefficient of variation) = $\frac{\text{standard deviation}}{\text{mean}} \times 100.$

Table 3. *Vitamin A in goat's milk*

(6-8 weeks post partum.)

Goat no.	Milk yield (ml./2 days)	Fat (%)	Vitamin A		Ester* (%)
			(i.u./100 ml.)	(i.u./g. fat)	
1	2670	3.1	174	56.0	98.5
2	1940	4.1	120	29.2	97.9
3	1330	3.8	128	33.7	98.6
4	2270	3.0	142	47.2	98.1
5	4600	4.3	165	38.3	99.2
6	1780	4.2	151	35.9	97.8
7	2730	4.0	110	27.5	98.9
8	3760	3.4	150	44.2	98.2
Mean	2635	3.7	142	39.0	98.4
c.v.	41.0	13.6	15.4	24.7	0.5

* The figures in this column show the proportion of the total vitamin A which was present as ester, and indicate that no measurable amount of vitamin A was present in the goat's milk.

Table 4. *Vitamin A in liver and kidneys and β -carotene in liver and colostrum of goats*

Goat no.	Cause of death and date	Thyroxine (mg./day)	Liver		Kidney vitamin A (i.u./g.)	β -Carotene in the first colostrum (μ g./100 ml.)
			Vitamin A (i.u./g.)	β -Carotene (μ g./g.)		
4	Hyperthyroidism (1950)	10	369	0.34	38	28
8	Hyperthyroidism* (1950)	10	253	0.00	9	12
9	Unknown (1950)	None	471	0.67	81	35
10	Bloat (1950)	None	452	0.09	77	19
5	† (1951)	3	176	0.84	8	46
7	† (1951)	3	272	Trace	33	24

* This animal received also 1 mg. stilboestrol per day.

† Killed after treatment with 3 mg. DL-thyroxine per day.

The occurrence of β -carotene along with vitamin A in the colostrum and liver of the goat

Cow colostrum is richer in carotene than the milk which is secreted later. It was noticed in the present experiments that the first colostrum of the goats was orange-yellow in colour. The immediately following samples of the secretion were yellow and were succeeded by the normal white milk of the goat. By analogy with the cow it was thought that yellow pigment of colostrum should contain β -carotene. Chromatography followed by spectrophotometry showed that the pigment contained

β -carotene. The amount in the colostrum varied from goat to goat as shown in Table 4. It was further thought that this colostrum carotene probably came from the goat's liver. Accordingly, the livers of six goats were examined post-mortem for β -carotene. The results in Table 4 show that β -carotene was demonstrable in most of the livers, and that the amount in the colostrum was probably determined by the amount in the liver. In view of the facts that the livers of goats examined in Liverpool by Goodwin & Gregory (1948) did not contain carotene and that one of the livers which we examined (Table 4) also contained none, the yellow pigment of

the liver was subjected to chromatography on alumina and then measured spectrophotometrically. In Fig. 1 the absorption spectra of a sample of the pigment from goat liver and of a sample of β -carotene (British Drug Houses Ltd.), both purified by chromatography on alumina, are shown. The figure demonstrates that the maxima of absorption for both the pigment from the goat liver and pure β -carotene were 423, 451 and 475 $m\mu$. in *n*-hexane. These maxima are concordant with those quoted by Karrer & Jucker (1950) which were also measured in *n*-hexane. They also agree closely with the values obtained in petrol solution by Morton (1942). The absorption spectrum of β -carotene isolated from goat colostrum had the same absorption maxima but has been omitted for the sake of brevity. These figures leave little doubt that the pigments of both goat liver and goat colostrum contain β -carotene.

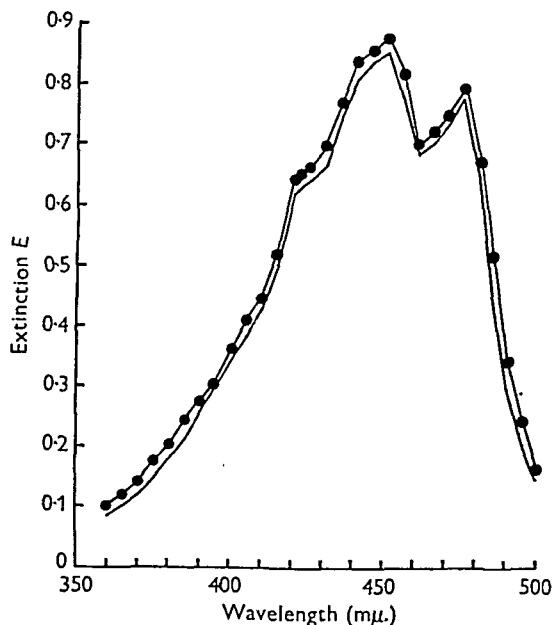


Fig. 1. Absorption spectrum of β -carotene and of the pigment isolated from goat's liver. Both pigments were dissolved in *n*-hexane after purification by chromatography on alumina. ●—●, pigment from goat's liver (goat no. 5); —, pure β -carotene.

In both liver and colostrum the β -carotene was accompanied by another yellow pigment which from its chromatographic properties on alumina, and by analogy with cow's milk was presumed to be lutein (xanthophyll), but this latter pigment was not investigated spectrophotometrically. Our goats therefore resembled the ewes investigated by Ganguly & Deuel (1951) which also had both carotene and lutein in the liver. The kidneys differed from the liver (Table 4) in not containing carotenoids or other yellow lipochromes. The figures in Table 4 show that the livers and kidneys

of the goats which had been treated with thyroxine contained less vitamin A than those of the untreated animals, nos. 9 and 10, in which the hepatic vitamin A was of the same order as that of the sheep examined by Moore & Payne (1942). The varying liver reserves listed in Table 4 will be referred to again later.

One of the goats was rather exceptional, for, within 2 days of parturition, the lactation resulting from the previous parturition had not completely ceased. The milk (which is obviously not to be confused with the preparturient colostrum which may, for experimental purposes, be expressed from the cow's udder), did not contain carotene or any other carotenoid. When this same goat came again into milk after parturition the new secretion was colostrum and was yellow. The presence of β -carotene in this colostrum was also confirmed by chromatography followed by spectrophotometry.

The effect of thyroxine and thiouracil on the carotene in cow's milk (Experiment 1)

From Figs. 2 and 3 and Table 5 it will be seen that in period 1 the removal of carotene from the pre-experimental diet was followed by a drop in the yield of carotene in the milk and in the percentage of carotene in the milk fat of all the cows. That this drop was more precipitate than the natural decline of the yield of milk is shown by the fact that in all cases the concentration of carotene in the milk decreased after the institution of the carotene-free diet. In period 2, when carotene was replaced in the diet, the trends of the yield of carotene and percentage of carotene in the milk and the trend of carotene concentration in the milk fat were all reversed.

In period 3, carotene in the milks of all the cows decreased in much the same way as it had already done in period 1. In period 4 the two control cows behaved, with respect to carotene, much as all six cows had done in period 2, for carotene in their milk increased. In the two cows which received thyroxine in period 4 the increases in the yield of carotene per cow were noticeably larger than the increases shown by the control cows. In the two cows which were treated with thiouracil no such increases occurred at all. The carotene content of the milk fat increased more rapidly in the two thyroxine-treated cows than in the two controls. Thiouracil had the reverse effect. The effect of thyroxine in facilitating the transfer of carotene to the milk is shown by the fact that in both the thyroxine-treated cows the concentration of carotene in the milk increased at the same time as the yield of milk was increasing.

In period 5, when all the cows continued to ingest carotene, the effects of the discontinuance of the two drugs on carotene in the milk were observed. The

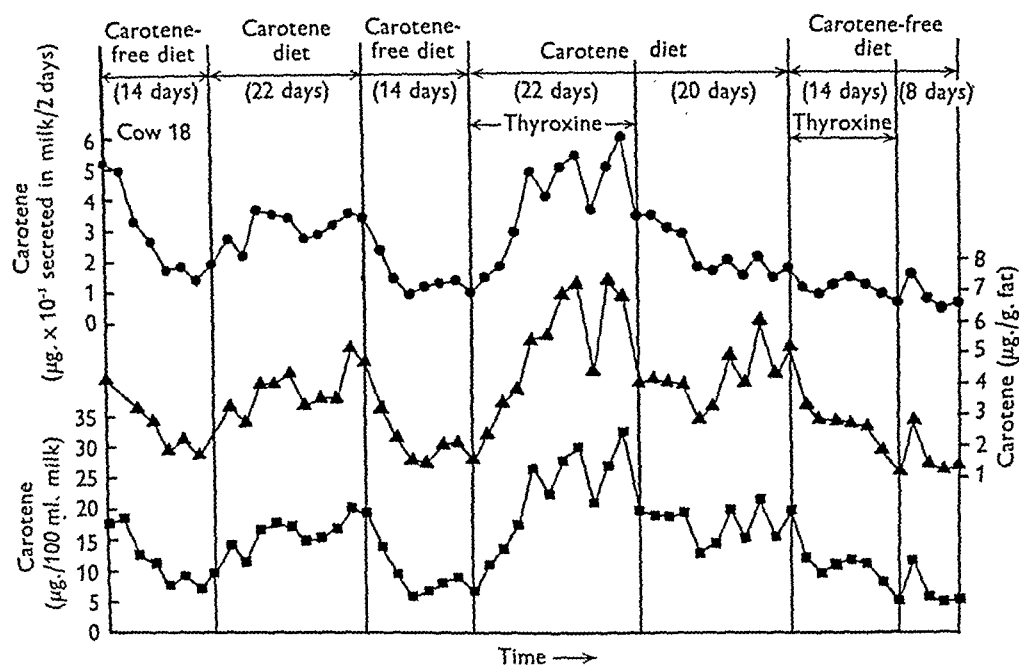


Fig. 2. The effect on the carotene of cow's milk of superimposition of treatment with thyroxine upon diets with and without carotene.

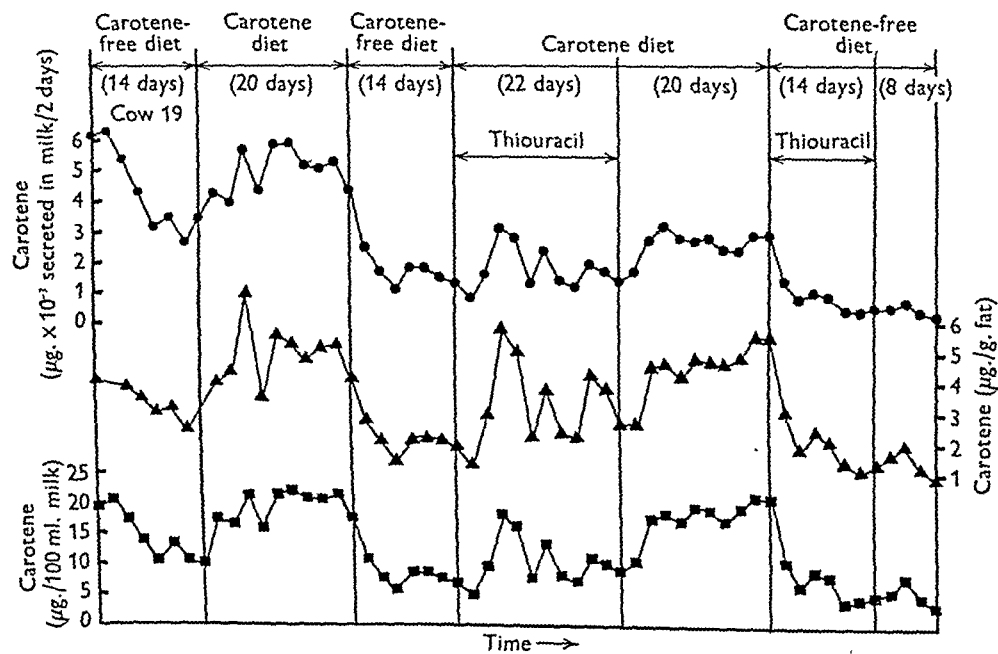


Fig. 3. The effect on the carotene of cow's milk of superimposition of treatment with thiouracil upon diets with and without carotene.

Table 5. *Effect of thyroxine and thiouracil on the mean content and the mean yield of β -carotene in the milk of cows both with and without β -carotene in the diet. Experiment 1*

(For details of dietary regimen see p. 404.)

Cow no.	Period	Treatment in periods 4 and 6	β -Carotene intake (mg./2 days)	Fat in milk (%)	β -Carotene in the milk		
					$\mu\text{g./100 ml.}$	$\mu\text{g./g. fat}$	$\mu\text{g.} \times 10^{-3}$ secreted/2 days
15	1	—	Trace*	4.2	15.1	3.6	4.2
	2	—	978	3.9	17.5	4.5	4.8
	3	—	Trace	3.6	9.3	2.6	2.3
	4	None	978	3.9	15.0	3.8	3.4
	5	—	948	3.8	16.5	4.3	4.1
	6	None	Trace	3.7	11.1	3.0	2.3
	7	—	Trace	3.6	2.9	0.8	0.6
16	1	—	Trace	4.1	9.0	2.2	2.4
	2	—	948	3.6	14.8	4.1	3.9
	3	—	Trace	3.7	10.1	2.7	2.5
	4	None	948	3.8	12.8	3.4	3.5
	5	—	972	4.0	15.7	3.9	3.5
	6	None	Trace	3.8	6.6	1.7	1.4
	7	—	Trace	4.0	4.2	1.1	0.8
17	1	—	Trace	3.9	12.7	3.3	3.6
	2	—	912	3.8	17.4	4.6	4.0
	3	—	Trace	3.5	7.7	2.2	1.5
	4	Thyroxine	960	4.2	23.6	5.6	5.4
	5	—	960	4.4	18.8	4.3	3.6
	6	Thyroxine	Trace	4.4	8.9	2.0	1.5
	7	—	Trace	4.3	3.6	0.8	0.8
18	1	—	Trace	4.0	11.8	3.0	2.9
	2	—	966	4.2	16.2	3.9	3.2
	3	—	Trace	4.1	8.2	2.0	1.3
	4	Thyroxine	984	4.3	22.5	5.2	4.0
	5	—	954	4.2	17.5	4.2	2.2
	6	Thyroxine	Trace	4.0	9.7	2.4	1.0
	7	—	Trace	4.1	7.2	1.8	0.9
19	1	—	Trace	4.2	15.1	3.3	4.6
	2	—	972	4.1	18.8	4.6	4.9
	3	—	Trace	3.8	7.9	2.1	1.7
	4	Thiouracil	984	3.4	10.6	3.1	1.9
	5	—	930	3.9	17.0	4.4	2.6
	6	Thiouracil	Trace	3.6	6.7	1.9	0.8
	7	—	Trace	3.9	6.1	1.6	0.5
20	1	—	Trace	4.2	12.1	2.9	3.4
	2	—	930	3.8	15.4	4.1	4.0
	3	—	Trace	3.6	8.7	2.4	2.0
	4	Thiouracil	978	3.9	8.2	2.1	1.5
	5	—	972	3.4	14.1	4.1	2.4
	6	Thiouracil	Trace	3.3	9.5	2.9	1.5
	7	—	Trace	3.5	3.5	1.0	0.5

* Approx. 2 mg./2 days from the straw.

concentration of carotene in the milk was maintained in each of the two control animals, whilst in the two which had received thyroxine it showed a marked diminution. By contrast, the two which had received thiouracil showed increases in carotene yield and in the percentage of carotene in both milk and fat. As was expected, neither thyroxine nor thiouracil appeared to affect the carotene content of the milk produced during periods 6 and 7 when the cows were receiving no carotene in their diet.

Effect of thyroxine and thiouracil on vitamin A in cow's milk (Experiment 1)

In all six cows, replacing the pre-experimental diet by one not containing carotene was followed by a fall in the yield of vitamin A in the milk, and this fall was greater than the natural decline of the milk yield because the concentration of vitamin A in the milk was also decreased (Figs. 4, 5 and Table 6). The vitamin A per g. of fat was likewise decreased in all six cows. All these three effects were reversed when

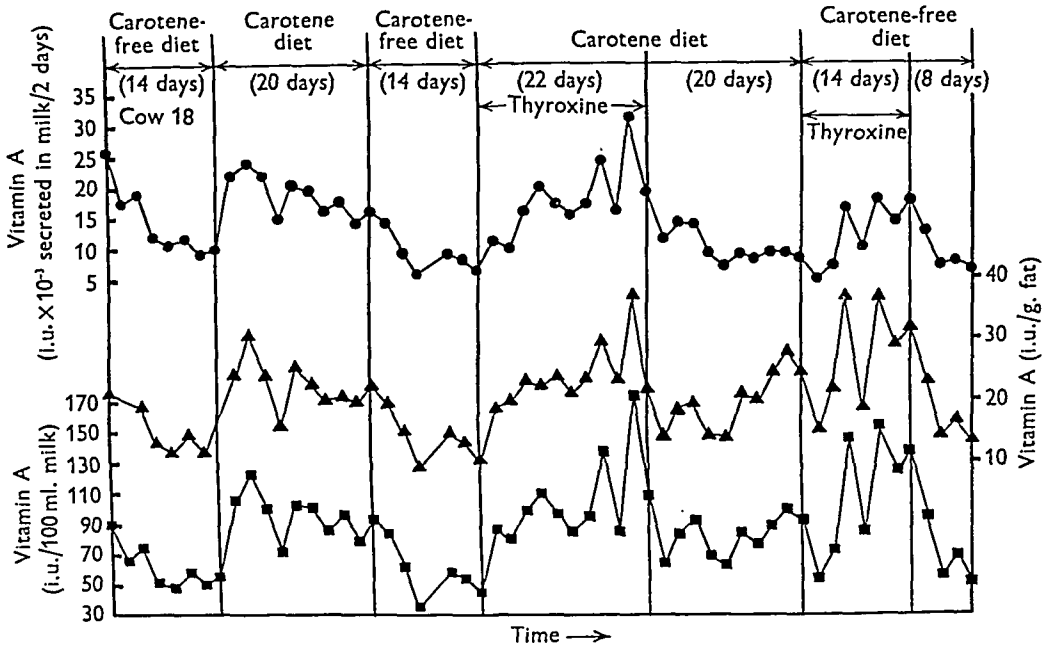


Fig. 4. The effect on the vitamin A of cow's milk of superimposition of treatment with thyroxine upon diets with and without carotene.

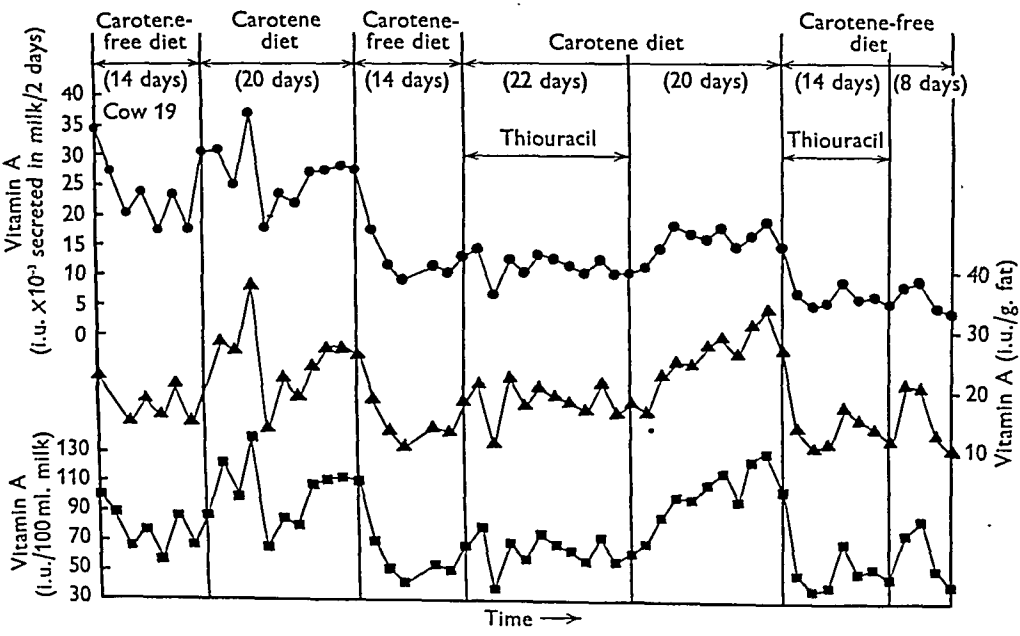


Fig. 5. The effect on the vitamin A of cow's milk of superimposition of treatment with thiouracil upon diets with and without carotene.

the cows returned in period 2 to a diet containing carotene. The increases in the vitamin A per g. fat and per 100 ml. milk were very marked in period 2. In period 3, when carotene was again withheld, a repetition of the results obtained in period 1 was observed, except that some of the cows reverted to figures for milk vitamin A which were less than those of period 1.

vitamin A in the milk of the two control cows were much enhanced by reversion to the carotene-containing diet in period 4. These increases were magnified in cows treated with thyroxine and nullified in cows treated with thiouracil.

The removal of thyroxine while the cows were still ingesting carotene (period 5) caused a sudden fall of vitamin A in both milk and fat and a sudden fall of

Table 6. *Effect of thyroxine and thiouracil on the mean content and the mean yield of total vitamin A and on the partition of vitamin A in the milk of cows both with and without β -carotene in the diet. Experiment 1*

(For details of dietary regimen see p. 404.)

Cow no.	Period	Treatment in periods 4 and 6	β -Carotene intake (mg./2 days)	Vitamin A in the milk				
				i.u./100 ml.	i.u./g. fat	Ester (%)	Alcohol (%)	i.u. $\times 10^{-3}$ secreted/2 days
15	1	—	Trace*	64	15.3	89	9.3	18.3
	2	—	978	90	26.3	91	6.8	24.7
	3	—	Trace	60	16.8	90	9.2	14.8
	4	None	978	72	18.7	93	7.2	16.4
	5	—	948	70	18.6	91	6.1	15.8
	6	None	Trace	52	14.1	89	8.2	10.9
	7	—	Trace	52	14.4	89	11.3	10.5
16	1	—	Trace	59	13.8	91	6.3	15.8
	2	—	948	77	21.7	94	4.4	20.2
	3	—	Trace	51	13.4	89	8.0	12.5
	4	None	948	70	18.4	93	4.7	16.0
	5	—	972	70	17.5	94	4.0	15.7
	6	None	Trace	45	11.8	89	8.6	9.6
	7	—	Trace	48	12.1	87	10.6	11.4
17	1	—	Trace	69	17.1	90	7.1	19.3
	2	—	912	88	23.2	93	5.5	20.7
	3	—	Trace	54	15.2	91	8.3	10.8
	4	Thyroxine	960	103	24.2	93	5.5	21.9
	5	—	960	80	18.6	94	4.9	15.2
	6	Thyroxine	Trace	95	21.2	83	15.1	26.3
	7	—	Trace	53	12.2	87	11.2	10.8
18	1	—	Trace	62	15.4	91	6.4	15.3
	2	—	966	95	22.6	94	4.4	18.4
	3	—	Trace	55	13.5	90	7.8	8.7
	4	Thyroxine	984	104	24.1	94	4.4	17.8
	5	—	954	83	20.0	94	3.9	10.5
	6	Thyroxine	Trace	110	27.2	80	18.3	12.4
	7	—	Trace	73	18.0	88	9.1	9.0
19	1	—	Trace	79	18.1	91	7.4	23.6
	2	—	972	102	25.5	91	6.3	27.0
	3	—	Trace	55	14.5	91	7.7	12.0
	4	Thiouracil	984	62	18.3	89	8.6	11.1
	5	—	930	98	25.4	92	6.2	15.0
	6	Thiouracil	Trace	49	13.4	90	7.3	5.8
	7	—	Trace	70	18.4	88	8.1	6.6
20	1	—	Trace	66	15.5	92	7.4	18.5
	2	—	930	95	27.6	92	5.5	24.2
	3	—	Trace	53	14.4	90	7.2	11.9
	4	Thiouracil	978	61	15.9	89	8.0	11.4
	5	—	972	92	27.7	93	5.1	16.0
	6	Thiouracil	Trace	45	13.7	87	8.2	7.0
	7	—	Trace	36	10.2	89	8.9	5.0

* Approx. 2 mg./2 days from the straw.

In period 4 the effects of the hormonal treatments were evident. Thyroxine caused a very marked rise in the vitamin A per g. fat and per 100 ml. milk of both the cows which were treated with it, while the behaviour of the two cows which received thiouracil resembled that of the two controls. The yields of

the vitamin A yield. Discontinuance of thiouracil caused effects of the reverse kind which were, however, not quite so marked (Fig. 5).

An interesting feature of Tables 5 and 6 is the effect, on the concentration of carotene and vitamin A in the fat, of discontinuance of thiouracil while

carotene was being ingested. This effect was strikingly similar to that of giving thyroxine. Conversely, it is true to say that the effect of discontinuing thyroxine in cows nos. 17 and 18 was similar to that of giving thiouracil to cows nos. 19 and 20.

Partition of vitamin A between alcoholic and ester forms in cow's milk

Fig. 6 and Table 6 show that when, during periods 1, 3, 6 and 7 the cows were deprived of carotene, the proportion of vitamin A present in the

The vitamin A in goat's milk was found to be all in the esterified form, so that free vitamin A was absent. For this reason, therefore, and also because the goats were never deprived of carotene, no attempt was made to partition vitamin A in this experiment. In general the effects were similar to those already reported for total vitamin A in cow's milk. Thus thyroxine caused an increase in the vitamin A both per 100 ml. milk and per g. fat, even although treatment with thyroxine caused an increase of the percentage of fat in the milk (Fig. 7 and Table 7). In goat no. 3, which received thy-

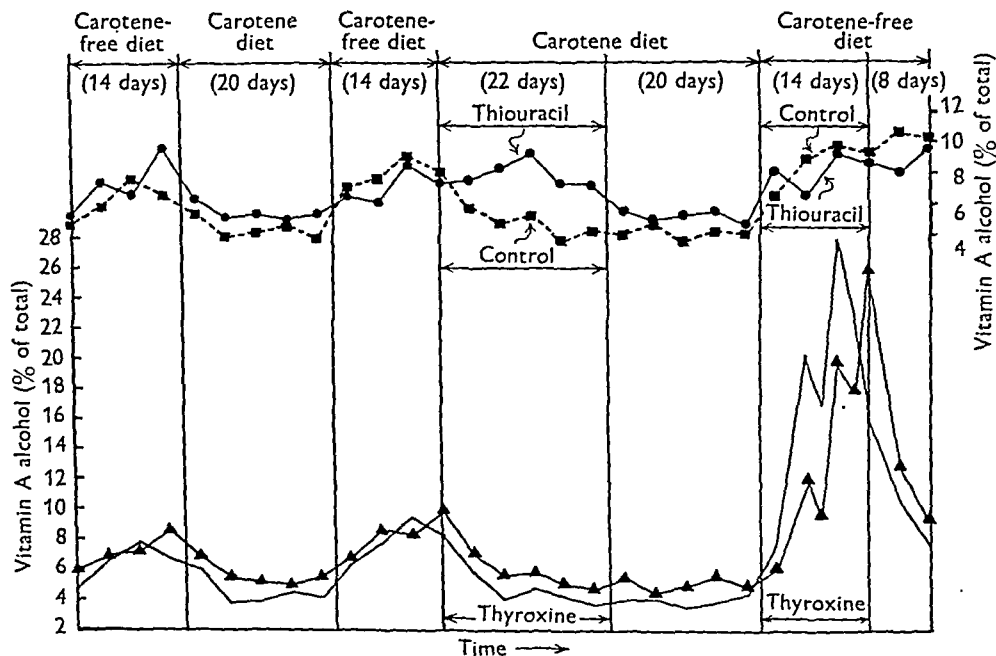


Fig. 6. The effect on the vitamin A alcohol of cow's milk of superimposition of thyroxine treatment (lower two curves) upon diets with and without carotene. Of the two upper curves, one shows the effect of dietary treatment alone and the other shows the lack of effect of superimposition of thiouracil treatment upon each diet. ●—●, cow no. 20; ■—■, cow no. 16; ▲—▲, cow no. 17; —, cow no. 18.

milk in the alcohol form increased. When carotene was being ingested, reversion to the initial percentage occurred. During period 6, the two cows which received thyroxine simultaneously with a carotene-free diet exhibited very large increases in the proportion of vitamin A present in the form of alcohol in the milk. These increases were spasmodic and coincided with increases of the total concentration of vitamin A in the milk; in these cases most of the increase of the total vitamin A was due to the increase of the alcohol fraction.

Effects of thyroxine, thiouracil and stilboestrol on the vitamin A in goat's milk (Experiment 2)

Numerous analyses of milk from many goats confirmed the well known observation that carotenoids occur only in minute traces in goat's milk.

roxine alone, these increases were accompanied by a transient increase in the milk yield. In each goat which received thyroxine, whether with or without stilboestrol, there were marked increases in the vitamin A per 100 ml. milk and in the vitamin A per g. fat. Of two goats, nos. 4 and 8, which died of hyperthyroidism, one (no. 8) had received stilboestrol as well as thyroxine. In each of these two animals the secretion of the watery phase of the milk regressed much faster than the secretion of fat, so that average fat percentages for period 2 were 8.3 and 9.9 compared with 4.8 and 4.4 in the other two goats which received thyroxine. In goat no. 7 stilboestrol administration caused lactation to cease at the end of period 2 (Fig. 8), but a week later (6 days after the discontinuance of the stilboestrol) she came back into milk at the same yield as

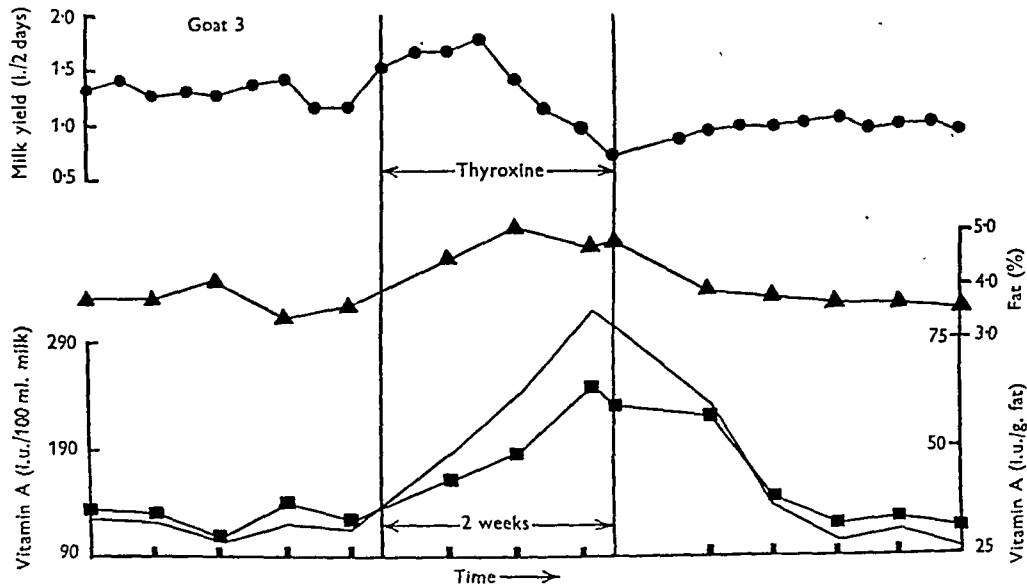


Fig. 7. Milk yield, percentage fat in the milk and the vitamin A content of milk and fat of a thyroxine-treated goat. —, vitamin A (i.u./100 ml. milk); ■—■, vitamin A (i.u./g. fat); ▲—▲, fat (%); ●—●, milk yield (l./2 days).

Table 7. *The effect of thyroxine, thiouracil and thyroxine plus stilboestrol on the mean fat and the mean vitamin A contents of goat's milk. Experiment 2*
(For details of dietary regimen see p. 405.)

Goat no.	Period	Treatment in period 2	Dietary carotene (mg./2 days)	Fat (%)	Vitamin A		
					i.u./100 ml. milk	i.u./g. fat	i.u. $\times 10^{-3}$ secreted/2 days
1	1	None	139	3.1	179	57	4.4
	2		124	3.3	172	53	3.9
	3		100	3.3	168	52	2.7
2	1	None	94	4.3	114	27	1.8
	2		82	4.6	107	23	1.2
	3		45	4.2	97	23	2.2
5	1	Thiouracil	143	4.1	163	40	7.3
	2		133	3.2	104	33	4.5
	3		123	3.6	108	30	4.1
6	1	Thiouracil	138	4.3	156	36	2.6
	2		109	3.8	107	30	1.5
	3		93	4.1	120	29	1.0
3	1	Thyroxine	122	3.7	119	32	1.6
	2		70	4.8	261	55	3.5
	3		109	3.7	136	36	1.3
4	1	Thyroxine	117	3.1	134	43	2.8
	2		40	4.4	225	51	1.3
	3		—	—	—	—	—
7	1	Thyroxine + stilboestrol	142	3.9	112	29	3.4
	2		87	8.3	285	34	5.5
	3		111	7.6	238	30	1.0
8	1	Thyroxine + stilboestrol	114	3.4	138	41	3.1
	2		12	9.9	441	45	2.2
	3		—	—	—	—	—

previously. In this goat, as she rapidly dried off, both the percentage of fat in the milk and the percentage of vitamin A in the milk increased rapidly. When she resumed lactation both the fat and vitamin A were of about the same magnitude as they had been when lactation ceased (Fig. 8). Neither when she ceased nor when she resumed milking did this goat show any evidence of carotenoids in the milk.

supposed, from the work of Hoch & Hoch (1946), to be in the alcohol form. However, Chanda (1951) has found that cows at grass have some 16–30 % of their blood plasma vitamin A in the ester form. Glover, Goodwin & Morton (1947) have adduced evidence that reserves of vitamin A are present in the liver as the ester but are in equilibrium with vitamin A alcohol in the blood. On their hypothesis, if a cow is deprived of carotene and thereby forced to mobilize

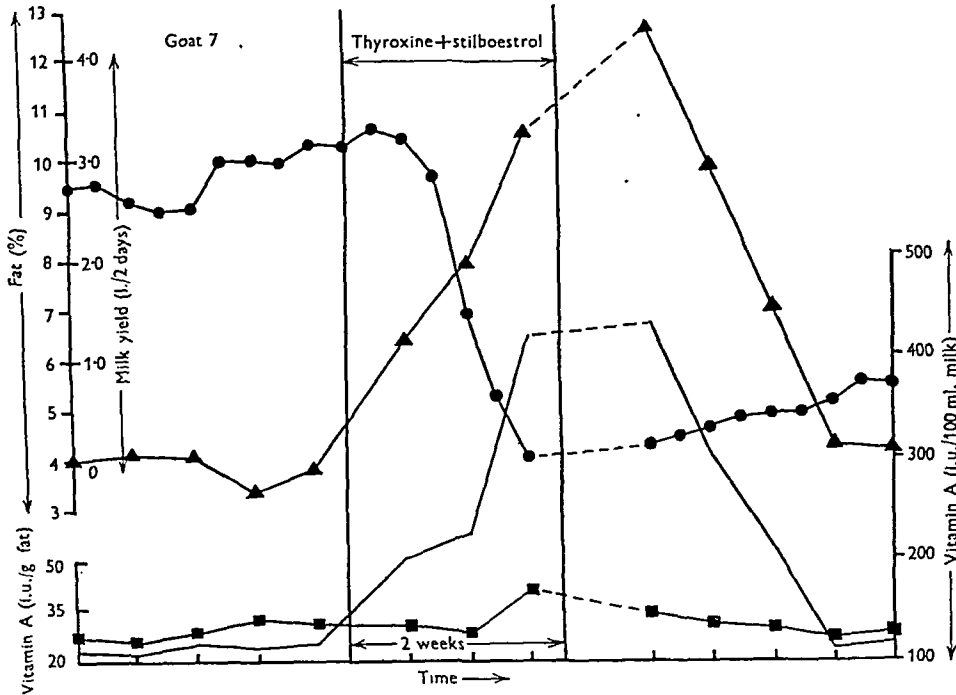


Fig. 8. Milk yield, percentage fat in the milk and the vitamin A content of milk and fat of a goat treated with thyroxine and stilboestrol. The breaks in the curves indicate that the treatment completely inhibited lactation just before the end of period 2 but that it was resumed in period 3. —, vitamin A (i.u./100 ml. milk); ■—■, vitamin A (i.u./g. fat); ▲—▲, fat (%); ●—●, milk yield (l./2 days).

Thiouracil depressed the milk yield slightly, but at the same time it noticeably reduced the percentage of fat in the milk. This drug also depressed the amount of vitamin A per g. fat so that the yield of vitamin A in the milk was depressed (Table 7).

DISCUSSION

The results of the present experiments may be explained by supposing that when dietary carotene is absorbed it is transformed into the ester form of vitamin A (Thompson *et al.* 1950) and passes via the lymph, the thoracic duct and the blood stream to the mammary gland. In the cow some carotene probably accompanies the vitamin A ester, since both will be constituents of the chylomicrons. In the goat the accompanying carotene will be negligible. Most of the vitamin A in the blood is usually

its hepatic reserves, an increase in vitamin A alcohol in the milk would not be unexpected and this is what was found in the present experiments. Experiments by Greeves & Schmidt (1936) have shown that thyroxine accelerates the release of reserves of vitamin A from the livers of rats kept on diets deficient in the vitamin, thus explaining the effect of thyroxine in the present experiments in causing enhanced increases of vitamin A alcohol in the milk when, in period 6, the cows were deprived of carotene. The fact that vitamin A in the milk of cows was less when thiouracil was given with a carotene-free diet than it had been on the same diet without thiouracil (Table 6), can be explained on the basis of the observations of Kelley & Day (1948) who found thiouracil to retard the rate of depletion of hepatic stores of vitamin A in rats on a diet deficient in the vitamin.

Johnson & Baumann (1947) showed that, in rats, thyroxine greatly enhanced hepatic storage of vitamin A from dietary carotene but had little effect on storage from preformed dietary vitamin A. They also found that thiourea inhibited storage from dietary carotene but not from dietary vitamin A. It is therefore reasonable to suppose that the effect of thyroxine in the cow in stimulating the apparent digestibility of carotene (Chanda *et al.* 1951*b*) was due rather to the stimulation by the hormone of conversion of carotene to vitamin A in the intestinal mucosa (Glover *et al.* 1948; Thompson, Ganguly & Kon, 1949) than to any effect it may have had in increasing the absorption of carotene as such. Nevertheless, the figures in the last column of Table 5 do give reason for supposing that the increase caused by thyroxine in the output of milk carotene itself was chiefly from dietary carotene. Thus, in the control cows 15 and 16, the output of carotene in the milk was smaller in period 4 than in period 2, both of these being carotene periods. When thyroxine was given in period 4, however, cows 17 and 18 show that thyroxine caused the carotene output to be bigger in period 4 than in period 2. That this was an effect of thyroxine is shown by its reversal in cows nos. 19 and 20 which received thiouracil in period 4. That these increases in carotene output were probably of dietary rather than hepatic origin is shown by comparing periods 3 and 6 when no carotene was fed. In these circumstances thyroxine failed to cause any change in the output of milk carotene.

In this discussion it is, of course, assumed throughout that carotene, once it has passed into the lymph from the intestine, is of no further use to the cow as a source of vitamin A. It has long been known (Drummond & McWalter, 1933) that injected carotene is not converted to vitamin A by the rat and, indeed, Sexton, Mehl & Deuel (1946) have had rats dying of vitamin A deficiency while their livers contained much β -carotene resulting from injection of it. In the goat only traces of carotene could have been absorbed in these experiments (Tables 3, 4 and 7), so that the effect of thyroxine in increasing the apparent digestibility of carotene in these animals (Chanda *et al.* 1951*b*) may reasonably be attributed to an increase in the rate of conversion of carotene to vitamin A.

A considerable contribution to the vitamin A in the milk of the goats which were treated with thyroxine was probably made by their liver reserves after the overlarge dose of thyroxine had, by adversely affecting their appetites (Table 7), caused their carotene intakes to be reduced. The magnitude of this hepatic contribution can be judged by comparing, in Table 4, the liver reserves of the treated and untreated goats.

Tables 2 and 3 show that the goat's milk was

richer in vitamin A than the cow's milk and that the goat's milk unlike the cow's milk did not contain either carotene or vitamin A alcohol. The greater amount of vitamin A in goat's milk can perhaps be related to the more active thyroid gland of the goat as shown in Table 8. In that table data on both goats and cows from the present experiments and from those of Chanda *et al.* (1951*a*) are presented together with the data of Schultze & Turner (1945) on the rates of secretion of thyroxine by cows and goats. The table suggests that the greater apparent digestibility of carotene by goats is associated with both a higher vitamin A content of the milk and with a greater thyroid activity. The greater vitamin A content of the goat's milk could not have been a dietary effect because, as Table 8 shows, the

Table 8. *The rate of secretion of thyroxine, the digestibility of carotene and the secretion of vitamin A in the milk of cows and goats*

	Cows	Goats
Average body weight (kg.)	450	35
Average carotene intake (mg./day)	496	42
Carotene intake (mg./kg. body weight)	1.1	1.2
Apparent digestibility of carotene (%)*	56	63
Rate of secretion of thyroxine (mg./kg. body weight)†	22	34
Vitamin A in milk (i.u./g. fat)	21‡	39

* Data of Chanda *et al.* (1951*a*).

† Data from Schultze & Turner (1945).

‡ This value does not include the carotene.

carotene intakes of the cows and goats before hormone treatment were practically equal when calculated in terms of body weight. The larger concentration of carotene in the milk from cows treated with thyroxine leaves the observation of Fellenberg & Grüter (1932), that thyroidectomized goats secreted yellow milk, unexplained. Indeed, in the present experiments, goats treated with either thyroxine or thiouracil showed no evidence of the presence of carotenoids in the milk. Nevertheless, β -carotene was demonstrated in goat's colostrum (Table 4). It therefore seems that there is some hormonal influence on the goat's mammary gland at parturition which causes β -carotene regularly to appear together with other carotenoids in the colostrum. The appearance of carotene in goat's colostrum could hardly have been due to the thyroid which does not attain maximum activity until the peak of lactation at which time the goat's milk is free from carotenoids. Neither do oestrogens appear to be responsible for the occurrence of carotene in goat's colostrum. Thus goat no. 7 (Fig. 8), which resumed lactation after having been 'dried off' by stilboestrol, produced milk free from

carotenoids in the resumed lactation. Similarly, another goat which was giving some carotene-free milk 2 days before parturition, gave β -carotene along with other carotenoids in the post-partum mammary secretion. If, therefore, the occurrence of β -carotene in goat's colostrum is under hormonal control it does not seem from the present experiments that the hormones concerned are either thyroxine or oestrogens.

SUMMARY

The metabolism of carotene in six lactating cows and in eight lactating goats was investigated with the following results:

1. Goat's milk contained 142 i.u. vitamin A/100 ml., all of it in the ester form. Cow's milk contained 88 i.u. vitamin A, 6% of which was in the alcohol form. Unlike the cow's milk, that of goat did not contain carotenoids, but in the colostrum and in the liver of goats β -carotene was demonstrated.

2. When the cows were deprived of carotene for a fortnight their milk yields were not affected, but the concentrations of vitamin A and carotene decreased. At the same time the proportion of vitamin A present in the alcohol form increased.

Superimposing thyroxine treatment upon carotene deprivation caused even greater increases of vitamin A alcohol in the milk.

3. On re-instatement of the carotene-containing diet the concentrations of both carotene and vitamin A increased at rates which were enhanced by thyroxine and diminished by thiouracil. The effects on vitamin A and carotene of discontinuing thyroxine while the cows were still ingesting carotene were similar to those produced by injecting thiouracil. Conversely, the effects of discontinuing thiouracil were similar to those of giving thyroxine.

4. Thyroxine and thiouracil produced changes in the composition of goat's milk which were similar to those found in cow's milk.

The authors wish to thank Dr M. L. McNaught and Miss H. M. Clapham for their kind collaboration during a part of these experiments. The authors also wish to thank Miss P. McGuigan, Miss S. McLaughlan and Miss M. Millar for technical assistance. Thanks are also due to Dr W. Holmes for making cows from the Hannah Herd available for these experiments.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen

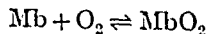
2. THE RELATION BETWEEN THE FIRST ORDER RATE CONSTANT AND THE PARTIAL PRESSURE OF OXYGEN

By P. GEORGE AND C. J. STRATMANN*
Department of Colloid Science, University of Cambridge

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In a previous paper (George & Stratmann, 1952) we showed that the oxidation of myoglobin to metmyoglobin by oxygen at constant oxygen pressure, pH, salt concentration and temperature was first order in unoxidized myoglobin. It thus resembles the autoxidation of haemoglobin investigated by Brooks (1931). In air the oxidation rate is 4.25 times faster than with haemoglobin.

Brooks (1935) showed that the observed first order rate constant for haemoglobin autoxidation was a function of the oxygen pressure, having a well defined maximum value at about 20 mm. In this paper the results of a similar investigation are reported for myoglobin, together with the determination of the equilibrium constant of the myoglobin-oxygen reaction, K_e .



under the conditions of the experiments. This value of the equilibrium constant is used in an analysis of the results based on a suggestion by George (1949) that the observed first order constant for haemoglobin was a complex function given by

$$k_{\text{obs.}} = k \frac{K}{K + [\text{O}_2]} \frac{[\text{O}_2]}{K + [\text{O}_2]}, \quad (1)$$

where K is the reciprocal of the equilibrium constant for the formation of oxyhaemoglobin. A full comparison is made between the kinetic data for myoglobin and haemoglobin.

MATERIALS AND APPARATUS

The myoglobin used in these experiments was prepared by the method described by George & Stratmann (1952). The myoglobin solutions were heavily buffered with K_2HPO_4 and KH_2PO_4 , giving a total concentration of phosphate ions of 0.6M with a pH of 5.69 at 30°.

The flow apparatus was similar to that described by Brooks (1935). The gas mixtures were prepared from cylinders of O_2 -free N_2 and either air or O_2 of 'medical' purity. The gases were passed first through cotton wool. The N_2 was passed through alkaline pyrogallol solution and the O_2 or air through alkaline permanganate solution. In order

to prevent deposition of moisture in the capillaries of the flow meters the gases were further washed in half-saturated NaCl. After passing through the flow meters the gases were mixed, passed through a glass coil immersed in the thermostat to preheat it, then passed through a buffer solution like that used for the myoglobin and finally led into the reaction vessel. A similar coil and tube containing buffer solution was used to cool the gas when the flask was immersed in the ice bath employed in the preliminary preparation of the MbO_2 described below. The rate of gas flow was 6 l./hr.

Kinetic measurements

The flask used in these experiments was similar to the one used by Brooks (1935) but was boat-shaped like a Meldrum-Roughton boat but without the central partition. A solution of metmyoglobin (MetMb) with a concentration of $2.57 \times 10^{-4}\text{M}$ was run into the flask. The optical density of the MetMb having been recorded, a small quantity of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was added and the flask gently shaken until the slight excess of $\text{Na}_2\text{S}_2\text{O}_4$ was oxidized and oxymyoglobin formed. The flask was then closed, placed in an ice bath and the gas mixture passed through the flask for 1.5–2 hr. whilst the flask was shaken slowly. A sample of the myoglobin solution was removed, diluted and the optical density measured. The usual sample removed was 1 ml. and diluted with 4 ml. of buffer solution. Details of the spectrophotometric methods of analysis used are given in a previous paper (George & Stratmann, 1952). The flask was then transferred to the thermostat at 30°. At intervals throughout the experiment samples were removed and the optical density measured. During the time taken to measure the optical density of the solutions there was no observable change due to autoxidation. The starting point of the reaction was taken as being the time when the flask was placed in the thermostat. Results were recorded as the percentage of oxymyoglobin present, taking the starting point of the reaction as 100% MbO_2 . Log percentage MbO_2 was plotted against time and the first order rate constant of the reaction ($k \text{ hr.}^{-1}$) was calculated from the slope of the line.

Equilibrium measurements

The oxygen dissociation curve of myoglobin was determined under the same general conditions as obtained in the kinetic experiments. The reaction flask had a side arm which was of such a size as to fit into the cell carrier of the spectrophotometer. The Pyrex glass used in its construction did not absorb appreciably in the range of wavelengths used, and since the method was a comparative one the small optical density difference between this tube and the reference cell was of no consequence.

* Present address: Department of Anatomy, University College, Gower Street, London, W.C. 1.

Two slightly different techniques were used. In the first a concentration of 5.14×10^{-5} M-myoglobin was used and the optical densities measured at 540 m μ . In the second, one-tenth of the above concentration was used and the optical densities measured at 420 m μ . In both methods approximately 10 ml. of metmyoglobin were placed in the flask. This was allowed to run into the side arm and the optical density

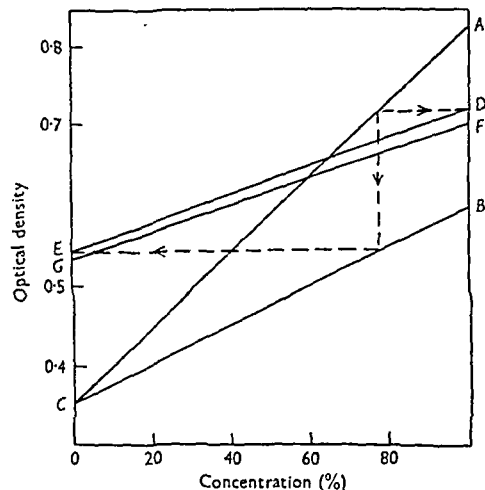


Fig. 1. Diagram used to facilitate calculation in the determination of the equilibrium constant of the reaction: $\text{Mb} + \text{O}_2 \rightleftharpoons \text{MbO}_2$ at 30° in 0.6M-phosphate buffer of pH 5.69. The points A, B and C represent the optical densities (at a wavelength of 540 m μ .) of solutions containing 100% MbO_2 , 100% Mb and 100% MetMb respectively. The line AC relates optical density to the percentage oxidation. The line BC relates optical density to the proportion of Mb and MetMb present in the solution if no MbO_2 is present. The point D gives the amount of MetMb formation which has occurred by autoxidation in the time taken for equilibration of the solution with the gas mixture. This point enables the line DE, which relates optical density to the proportion of Mb and MbO_2 present, after oxidation has been taken into account, to be drawn. The dotted line shows how the points D and E are derived. The measured optical density of the solution, equilibrated with the gas mixture, is used to determine the percentage of MbO_2 in the mixture by simply reading off the corresponding value on the line DE. The line FG represents a second equilibration experiment, using the same solution, when further oxidation has taken place.

was measured. The flask was removed from the spectrophotometer, placed in an ice bath and the solution run back into the body of the flask. A small quantity of $\text{Na}_2\text{S}_2\text{O}_4$ was added and the flask gently shaken until reduced myoglobin was formed. The optical density was again measured. The flask was again returned to the ice bath and shaken until all excess $\text{Na}_2\text{S}_2\text{O}_4$ was oxidized and oxy-myoglobin formed. The optical density of this solution was measured. In order to facilitate calculation a diagram shown in Fig. 1 was constructed which related optical density to the percentages of MbO_2 , Mb and MetMb present in the solution. A represents the optical density of a solution containing 100% MbO_2 , while B and C represent the optical densities of solutions containing 100% Mb and MetMb respectively.

Lines were drawn connecting A to C and B to C. The line AC relates the optical density of the solution with the proportion of MbO_2 and MetMb present in it or, in other words, the percentage oxidation. The line BC relates the optical density of the solution to the proportion of Mb and MetMb present in the mixture if no MbO_2 is present. A gas mixture of the desired partial pressure of O_2 was then passed through the flask which was shaken slowly in an ice bath for a period of 1 hr. The flask was then placed in the thermostat at 30° and shaken for 10–15 min. The flask was then quickly removed from the thermostat and the optical density of the solution measured. It was immediately replaced in the ice bath, opened to the air, the solution equilibrated with air and the optical density again measured. After equilibration with air the measured optical density (the point D on the diagram) gives the amount of oxidation of the mixture during the time taken for equilibration for the particular partial pressure of O_2 , and enables the line DE to be drawn, which relates the optical density of the solution with the proportion of Mb and MbO_2 present, after oxidation has been taken into consideration. The dotted line on the diagram shows how this point E is derived. The measured optical density of the solution equilibrated with the gas mixture can now be used to determine the percentage MbO_2 in the solution by simply reading off the value which corresponds to the measured optical density on the line DE. The whole cycle was repeated once or twice. The line FG represents a second equilibration experiment, using the same solution, when further oxidation had occurred.

In the second method the technique was essentially the same. A wavelength of 420 m μ . was chosen at which MetMb and Mb are isobestic and the diagram is in consequence simplified. As the solutions absorbed to a much greater extent at this wavelength it was necessary to use a solution approximately ten times more dilute than that used in the experiments described above.

Denaturation check

The azide-metmyoglobin check for denaturation was carried out at 2 mm. O_2 pressure, it having been found previously that at 152 mm. O_2 pressure no detectable denaturation occurred (George & Stratmann, 1952).

RESULTS

Denaturation check

In 3 hr. and at a partial pressure of 2 mm. of oxygen 77.5% of metmyoglobin was formed from oxy-myoglobin by autoxidation. Fig. 2 shows that some traces of denaturation of the protein are evident. This amounts, however, to no more than 1.5–1.7% of the myoglobin originally present, and it was not considered necessary to take this into consideration in the calculation of the rate constant.

Oxidation kinetics

The metmyoglobin formation was measured at 10 and 20 min. intervals and at many partial pressures of oxygen ranging from 0.3 to 760 mm. Fig. 3 shows four typical first order plots at the following oxygen pressures: 760, 1.0, 0.6 and 0.3 mm.

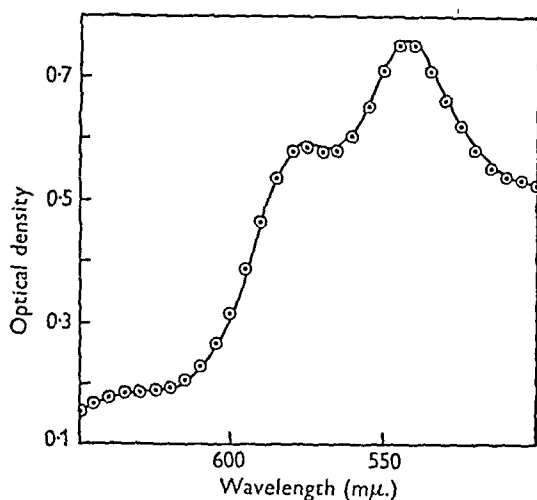


Fig. 2. Spectra of the azide-metmyoglobin complex formed before and after autoxidation of myoglobin at 30° in 0.6M-phosphate buffer of pH 5.69 at a partial pressure of oxygen of 2 mm. —, from a solution of metmyoglobin before autoxidation; ○, from a solution of metmyoglobin of the same concentration after autoxidation for 3 hr.

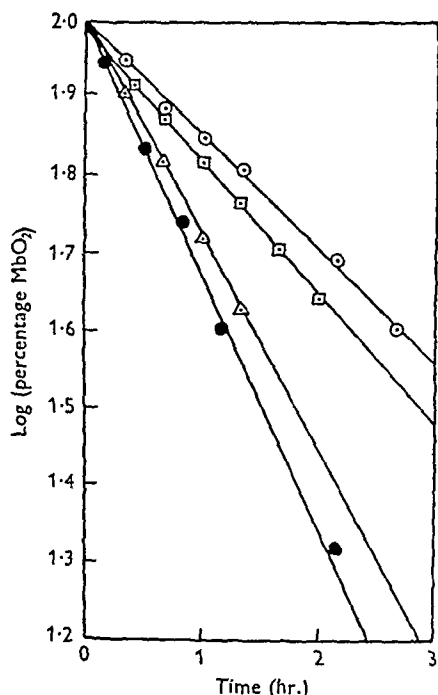


Fig. 3. Typical first order plots for the autoxidation of myoglobin to metmyoglobin in 0.6M-phosphate buffer of pH 5.69 at 30° at four different partial pressures of oxygen. ○, partial pressure of oxygen of 760 mm.; ●, partial pressure of oxygen of 1.0 mm.; △, partial pressure of oxygen of 0.6 mm.; □, partial pressure of oxygen of 0.3 mm.

The first order rate constants at these oxygen pressures were: 0.33, 0.75, 0.65 and 0.39 hr.⁻¹ respectively. Fig. 4 shows the relationship between the partial pressure of oxygen and the first order

rate constant over the entire range of oxygen pressures studied. A clearly defined maximum was observed at a partial pressure of about 1 mm. oxygen. Increase of the oxygen pressure above approximately 30 mm. was found to have very little effect on the rate constant, its value at these pressures being around 0.30 hr.⁻¹. All these results were obtained with the same sample of myoglobin.

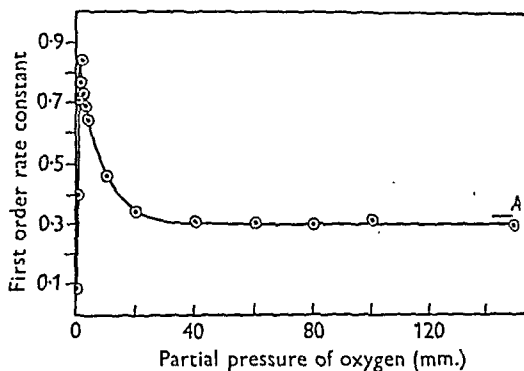


Fig. 4. The relationship between the partial pressure of oxygen and the first order rate constant for the autoxidation of myoglobin to metmyoglobin at 30° in 0.6M-phosphate buffer, over the entire range of oxygen pressures studied. A = the first order rate constant at 760 mm. p_{O_2} .

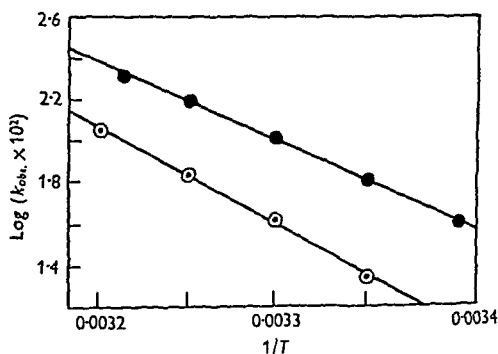


Fig. 5. Results of experiments to determine the activation energy of the autoxidation of myoglobin to metmyoglobin in 0.6M-phosphate buffer of pH 5.69 at 4 and 760 mm. partial pressures of oxygen. ●, partial pressure of oxygen = 4 mm.; ○, partial pressure of oxygen = 760 mm.

The activation energy of the autoxidation

The activation energy of the reaction was determined at two partial pressures of oxygen, namely 4 and 760 mm. A range of temperatures from 22 to 38° was studied and the rate constant was determined at 4° intervals over this range. Fig. 5 shows the results of these experiments. Logarithm of the rate constant is plotted against $1/T$. Calculations from this data gives the activation energy, E , for the reaction at 760 mm. oxygen pressure as 25.0 ± 1 kcal., and at 4 mm. as 19 ± 1 kcal. The temperature

coefficient of the buffer solution used in these experiments is -0.03 pH units for an increase of 10° (Cohn, 1927). The rate constant would be changed by this decrease of pH over 16° by about 10%, by analogy with Brooks's (1931) results with haemoglobin. If this is the case then the true values of the activation energies are within 1 kg.cal. of the above values.

It was found to be impossible to obtain a satisfactory value for the rate constant in an experiment carried out at 4 mm. oxygen pressure and at 38° owing to rapid denaturation of the protein at this temperature and oxygen pressure. It would appear that reduced myoglobin is more easily denatured by heat than is oxymyoglobin, since the experiment at 38° and 760 mm. oxygen pressure was reproducible and there were no detectable traces of denaturation.

The oxygen dissociation curve of myoglobin

The fraction of myoglobin in the form of oxymyoglobin at a given oxygen pressure was obtained by the method described above. Spectrophotometric analysis at $420\text{ m}\mu$. was used in addition to the measurements at $540\text{ m}\mu$. in the hope that more consistent results would be obtained. In Table 1,

Table 1. Measurement of equilibrium constant for $\text{Mb} + \text{O}_2$ at 30° in 0.6M-phosphate buffer, pH 5.69 by spectrophotometric analysis at 540 and $420\text{ m}\mu$.

Wavelength ($\text{m}\mu$.)	p_{O_2} (mm.)	Fraction MbO_2 present ($1 - \alpha$)	Mean $1 - \alpha$	$K_e = \frac{(1 - \alpha)}{\alpha p_{\text{O}_2}}$
540	0.6	0.37 0.38	0.38	1.02
540	1.0	0.55 0.53 0.48	0.55	1.08
540	2.0	0.60 0.62	0.61	0.78
420	3.0	0.65 0.67	0.66	0.65
420	5.0	0.84 0.81 0.78	0.81	0.85
420	10.0	0.95 0.90 0.85	0.90	0.90

Mean $K_e = 0.88 \pm 0.12\text{ mm.}^{-1}$

Therefore partial pressure of oxygen for half saturation = $1.00\text{--}1.32\text{ mm.}$, mean 1.16.

which records the results at a number of different oxygen pressures, it is seen that there is little to choose between the two methods. The value of the equilibrium constant K_e under the conditions of the experiments, at 30° , is 0.88 ± 0.12 from which it follows that the oxygen pressure for half saturation of the myoglobin is $1.00\text{--}1.32\text{ mm.}$

ANALYSIS OF THE RESULTS

The most striking feature of the results is that myoglobin, like haemoglobin, shows a maximum oxidation rate at low oxygen pressures. In the case of haemoglobin the oxygen pressure for the maximum rate corresponds closely to that required to half saturate the haemoglobin. This also holds for myoglobin as illustrated in Fig. 6.

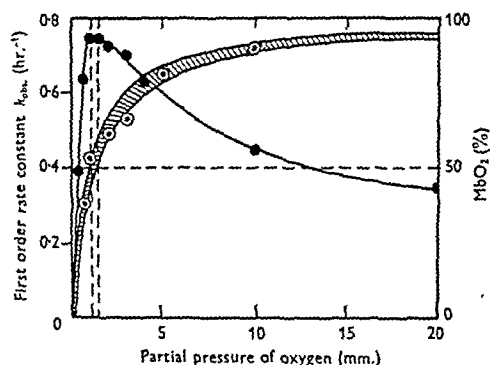


Fig. 6. The relationship between the oxygen pressure variation of the first order rate constant for the autoxidation of myoglobin to metmyoglobin and the oxygen dissociation curve of myoglobin at 30° in 0.6M-phosphate buffer of pH 5.69. ●, experimental values of the first order rate constant at partial pressures of oxygen over the range 0–20 mm. ○, experimental points in the determination of the oxygen dissociation curve of myoglobin. The true dissociation curve lies within the shaded area, the two lines being the limits of experimental error. ----, the partial pressure of oxygen required for half saturation of the myoglobin.

George (1949) suggested that the oxygen pressure function responsible for this behaviour in the case of haemoglobin was that given in eqn. 1.

$$k_{\text{obs.}} = k \frac{K}{K + [\text{O}_2]} \frac{[\text{O}_2]}{K + [\text{O}_2]} \quad (1)$$

Inspection of Fig. 4 shows that this equation alone cannot adequately account for the myoglobin data. At oxygen pressures above 30 mm., $k_{\text{obs.}}$ reaches a constant value and does not continuously diminish as p_{O_2} is increased in accord with this equation. It does, however, account for the maximum value of $k_{\text{obs.}}$ at the oxygen pressure required for half saturation of the myoglobin, which suggests that the correct function for $k_{\text{obs.}}$ may be of the form:

$$k_{\text{obs.}} = k_1 \frac{K}{K + [\text{O}_2]} \frac{[\text{O}_2]}{K + [\text{O}_2]} + Y \quad (2)$$

The additional oxygen pressure function Y can be numerically small at low oxygen pressures and in consequence $k_{\text{obs.}}$ can be given, in effect, by Eqn. 1 and, in addition, at high oxygen pressures Y can be such that a constant value for $k_{\text{obs.}}$ is obtained. The

actual form of Y in the present data can be found in the following way. $\frac{K}{K+[O_2]}$ and $\frac{[O_2]}{K+[O_2]}$ are the values of α and $(1-\alpha)$, the fractions of uncombined myoglobin and oxy-myoglobin respectively, so that eqn. 2 amounts to

$$k_{\text{obs.}} = k_1 \alpha(1-\alpha) + Y. \quad (3)$$

If k_1 can be evaluated then the variation of Y with oxygen pressure is given by $k_{\text{obs.}} - k_1 \alpha(1-\alpha)$. eqn. 3 may be rewritten

$$\frac{k_{\text{obs.}}}{\alpha(1-\alpha)} = k_1 + \frac{Y}{\alpha(1-\alpha)}.$$

The problem is thus one of choosing a suitable oxygen pressure function to plot $\frac{k_{\text{obs.}}}{\alpha(1-\alpha)}$ against so that k_1

may be obtained by extrapolation. Plotting against p_{O_2} itself is not satisfactory since a definite curve is obtained. The functions $(1-\alpha)/\alpha$ and $1/\alpha$ both give plots which are reasonably linear as shown in Fig. 7, the first point lying off the line in each case. Values of k_1 obtained from the intercept are 2.62 and 2.30 hr.⁻¹ respectively. Table 2 gives the calculation of Y based on these two values using eqn. 3. It can be seen that the variation of Y with oxygen pressure is of the type anticipated above. The linearity shown

in Fig. 7 when $\frac{k_{\text{obs.}}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ implies that Y is of the form $k_2(1-\alpha)^2$, and for the plot against $1/\alpha$ that Y is of the form $k_2(1-\alpha)$. It seems that the experimental data are not precise enough to decide between these two possibilities. This is understandable for the uncertainty in the velocity constant is about ± 0.02 hr.⁻¹ in each case. The slopes of the two lines are identical for their numerical value should be equal to $k_{\text{obs.}}$ at high oxygen pressures, hence $k_2 = 0.30$ hr.⁻¹. For myoglobin autoxidation we may thus summarize the data in the two possible expressions:

$$k_{\text{obs.}} = 2.62\alpha(1-\alpha) + 0.30(1-\alpha)^2, \quad (4a)$$

$$k_{\text{obs.}} = 2.30\alpha(1-\alpha) + 0.30(1-\alpha). \quad (4b)$$

Table 2. Calculations of Y , the high pressure component of the overall first order constant for myoglobin autoxidation

($Y = k_{\text{obs.}} - k_1 \alpha(1-\alpha)$, where $k_1 = 2.62$ or 2.30 hr.⁻¹
 $\alpha = K/(K + O_2)$, $1-\alpha = O_2/(K + O_2)$, where $K = 1.16$ mm.)

p_{O_2} (mm.)	$1-\alpha$	α	$\alpha(1-\alpha)$	$k_{\text{obs.}}$	$k_{\text{obs.}} - 2.62\alpha(1-\alpha)$	$k_{\text{obs.}} - 2.30\alpha(1-\alpha)$
0.3	0.21	0.79	0.166	0.39	(-0.07)	(-0.01)
0.6	0.35	0.65	0.228	0.635	0.035	0.105
1.0	0.47	0.53	0.249	0.745	0.085	0.165
1.5	0.57	0.43	0.245	0.74	0.100	0.180
2.0	0.64	0.36	0.230	0.74	0.130	0.200
3.0	0.725	0.275	0.199	0.70	0.180	0.240
4.0	0.78	0.22	0.172	0.635	0.205	0.245
100	0.99	0.011	0.011	0.32	0.29	0.29
160	0.99	0.007	0.007	0.30	0.28	0.28
760	1.00	0.0015	0.0015	0.33	0.33	0.33

It is of interest now to examine Brooks's (1935) data on haemoglobin autoxidation to see whether there is any evidence of similar behaviour. The analysis previously referred to is given in Table 3 (George, 1949). The ratio $\frac{k_{\text{obs.}}}{\alpha(1-\alpha)}$ is not in fact

constant but shows a small steady increase as p_{O_2} increases. The overall variation of this ratio, however, is far less than that obtained using Legge's

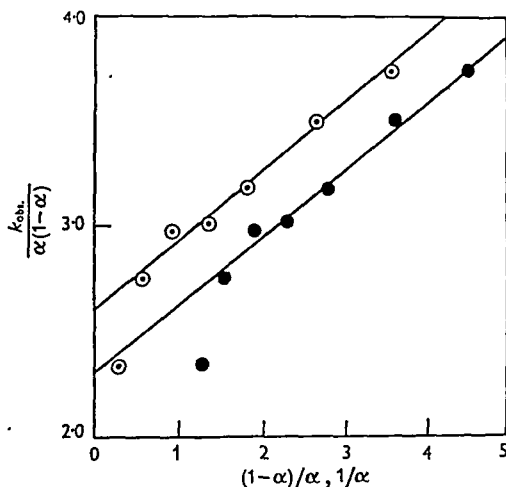


Fig. 7. Plot of $\frac{k_{\text{obs.}}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (\odot); and $1/\alpha$ (\bullet) in the case of myoglobin.

mechanism (Legge, 1942), assuming methaemoglobin formation by the unique decomposition of $Hb_4(O_2)_2$, and for this reason analysis of the results in terms of eqn. 1 was suggested. If now $\frac{k_{\text{obs.}}}{\alpha(1-\alpha)}$ is plotted against $(1-\alpha)/\alpha$ and $1/\alpha$ as has been done in Fig. 7 for myoglobin, the two lines in Fig. 8 are obtained, thus showing that haemoglobin autoxidation follows the same pattern. In this case the two possible equations are

$$k_{\text{obs.}} = 0.68\alpha(1-\alpha) + 4 \times 10^{-2}(1-\alpha)^2, \quad (5a)$$

$$k_{\text{obs.}} = 0.64\alpha(1-\alpha) + 4 \times 10^{-2}(1-\alpha). \quad (5b)$$

The points corresponding to the two lowest oxygen pressures do not lie on the lines, which may be attributed to uncertainty in the precise value for p_{O_2} ; a small uncertainty in this region leads to large

The empirical equation suggested by Brooks (1931)

$$k_{obs.} = k' \alpha \frac{bp}{1 + bp}$$

Table 3. Analysis of Brooks's (1935) results for haemoglobin autoxidation in 0.6M-phosphate buffer at 30°

(The observed first order constants are divided by the oxygen pressure function $\alpha(1-\alpha)$ based on equilibrium measurements on the haemoglobin-oxygen reaction under the same experimental conditions.)

p_{O_2}	$k_{obs.}$ (hr. ⁻¹ × 10 ²)	α	$1-\alpha$	$k_{obs.}/\alpha(1-\alpha)$
4.5	9.9	0.89	0.11	1.01
6.0	12.6	0.85	0.15	0.98
13.7	15.9	0.84	0.36	0.69
16.0	17.6	0.57	0.43	0.72
25.8	17.7	0.39	0.61	0.74
33.3	14.5	0.29	0.71	0.71
63.1	10.3	0.12	0.88	0.97
92.9	8.45	0.08	0.92	1.16
122	7.40	0.06	0.94	1.30
152	6.36	0.048	0.95	1.39
435	5.17	0.035	0.965	1.52
723	4.61	0.030	0.97	1.59

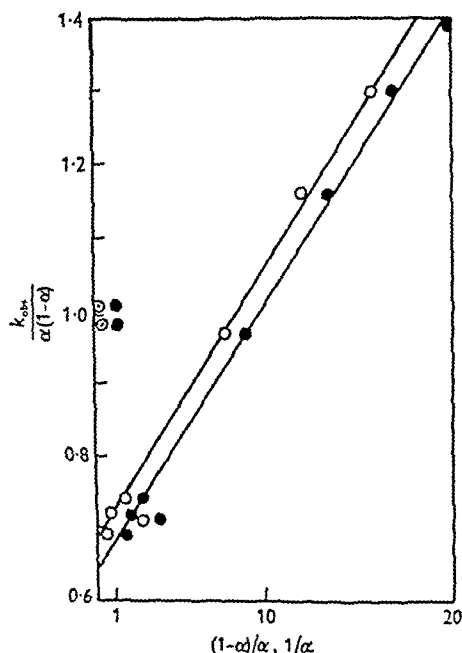


Fig. 8. Plot of $\frac{k_{obs.}}{\alpha(1-\alpha)}$ against $(1-\alpha)/\alpha$ (○) and $1/\alpha$ (●) in the case of haemoglobin. (From Brooks's (1935) data.)

discrepancies in the functions plotted. It again appears that the experimental data as a whole do not permit a choice between the two possible equations for $k_{obs.}$

proved very successful in accounting for the haemoglobin data, but it does not fit the myoglobin data so well as eqns. 4a and 4b at high oxygen pressures, for Brooks's equation like the single term $k_1\alpha(1-\alpha)$ in eqn. 1 diminishes continuously as p_{O_2} increases. The chief distinction between the haemoglobin and myoglobin systems is that the same range of oxygen pressures, 0.3–760 mm. covers a far wider range in the fraction of unoxxygenated haemoprotein in the case of myoglobin than in the case of haemoglobin. This is well brought out by comparison of Tables 2 and 3.

It is probable that other oxygen-pressure functions could be found to account equally well for $k_{obs.}$, particularly if more than one single function is used, as in eqn. 3 above. The merit of the present analysis is that it gives a close fit with the data using only oxygen-pressure functions directly connected with known possible reactants, i.e. α and $(1-\alpha)$ represent the fractions of unoxidized haemoprotein present in the reduced state and in combination with oxygen respectively.

DISCUSSION

The kinetic data on myoglobin and haemoglobin autoxidation reveal the same anomaly at low oxygen pressures. Eqns. 4a and 4b, 5a and 5b show that the observed rate constant shows a complex second order variation with oxygen pressure according to the product of the concentrations of Mb and MbO_2 , Hb and HbO_2 , whilst it is obtained from a first order reaction; for if Fe_p^{2+} represents the total ferrous protoporphyrin concentration and α and $(1-\alpha)$ the fractions of it free and combined with oxygen respectively, then the observed oxygen pressure relationship would be expected to derive from the kinetic equation

$$-\frac{dFe_p^{2+}}{dt} = k(1-\alpha) Fe_p^{2+} \alpha Fe_p^{2+},$$

i.e. second order in Fe_p^{2+} .

A possible explanation for this anomaly is that the autoxidation is a complicated sequence of reactions, such that the total concentration of unoxidized haemoprotein appears in the denominator of a rate equation of the form

$$\begin{aligned} -\frac{dFe_p^{2+}}{dt} &= \frac{k_a(1-\alpha) Fe_p^{2+} k_b \alpha Fe_p^{2+}}{k_c Fe_p^{2+}} \\ &= \frac{k_a k_b}{k_c} (1-\alpha) (\alpha) Fe_p^{2+}, \end{aligned} \quad (6)$$

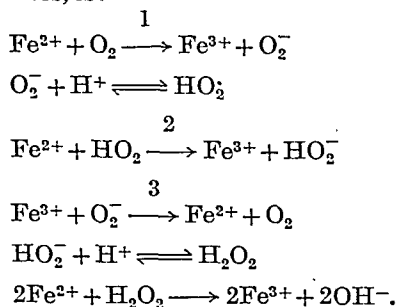
where k_a , k_b and k_c are the rate constants for certain steps in the mechanism. The numerator of the equation suggests that in step *a* MbO₂ or HbO₂ is involved, giving the term $k_a(1-\alpha) \text{Fe}_p^{2+}$ and in step *b* the free Mb or Hb, giving the term $k_b\alpha\text{Fe}_p^{2+}$.

This gives an indication as to what may be the operative rate equation at high oxygen pressures, whether it corresponds to 4*a* and 5*a* or 4*b* and 5*b*. As the oxygen pressure is increased Mb and Hb will decrease in concentration and MbO₂ and HbO₂ increase. Thus if both the free and oxygenated forms can undergo the same chemical reaction signified in the rate constant k_b , then the high oxygen pressure relationship would be given by

$$-\frac{d\text{Fe}_p^{2+}}{dt} = \frac{k_a(1-\alpha) \text{Fe}_p^{2+} k'_b(1-\alpha) \text{Fe}_p^{2+}}{k_c \text{Fe}_p^{2+}}, \quad (7)$$

and the ratio of k_b to k'_b would be given by 2.62/0.30 for myoglobin in eqn. 4*a* and by 0.68/0.04 in the case of haemoglobin in eqn. 5*a*. The fact that, with myoglobin, the activation energy at 760 mm. p_{O_2} is greater than that at 4 mm., as shown in Fig. 5, supports this interpretation, for when MbO₂ reacts additional energy required to break the iron-oxygen bond would be expected to appear as an increase in the activation energy. This is one simple way of accounting for the transition between the kinetics at low and high oxygen pressures. Attempts to account for the order of the reaction and the transition of the kinetics in terms of an equation of the form 4*b* and 5*b* have so far failed, and for this reason interpretation of the kinetics in terms of the rate eqns. 6 and 7 can profitably be explored.

Equations of this type are reminiscent of those derived from free radical mechanisms in which two valency states of a metal ion compete for the same free radical. The mechanism suggested by Weiss (1935) for the autoxidation of ferrous ion, in which the radicals O₂⁻, HO₂[·], H₂O₂ and the OH radical are intermediates, is:



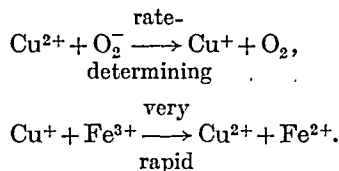
From the appropriate stationary state equations it can be shown that the oxidation rate should be

$$-\frac{d\text{Fe}^{2+}}{dt} = 4k_1\text{Fe}^{2+} \text{O}_2 \frac{k_2\text{Fe}^{2+}}{k_2\text{Fe}^{2+} + k_3\text{Fe}^{3+}}.$$

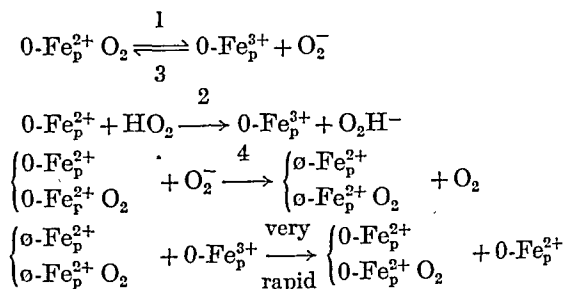
The numerator in this equation is similar to those in eqns. 6 and 7, but the denominator does not

correspond, in fact here the predicted rate is not first order with respect to the ferrous ion concentration. However, a free radical mechanism which can account for the myoglobin and haemoglobin kinetics can be derived in the following way.

Barb, Baxendale, George & Hargrave (1949, 1951), in an extensive study of the ionic iron-hydrogen peroxide system, showed that cupric ions are able to catalyse the reaction of Fe³⁺ with O₂⁻ through the two steps



Now if it is assumed that there is an auxiliary electron-accepting group in the unoxidized forms of myoglobin and haemoglobin that can act as the cupric ion does, this enables the following reaction scheme to be formulated:



followed by the reaction of the H₂O₂ with two more myoglobin or haemoglobin molecules. In this scheme 0-Fe_p²⁺, 0-Fe_p²⁺O₂ and 0-Fe_p³⁺ represent, for instance, Mb, MbO₂ and MetMb with the electron-accepting group in its oxidized state, *o*-Fe_p²⁺ and *o*-Fe_p²⁺O₂, when the group in Mb and MbO₂ has accepted an electron. The solution of the stationary state equations gives

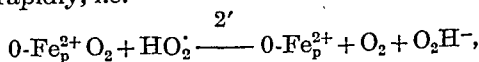
$$-\frac{d\text{Fe}^{2+}}{dt} = \frac{4k_1(1-\alpha) \text{Fe}_p^{2+} k_2\alpha\text{Fe}_p^{2+}}{k_4\text{Fe}_p^{2+} + k_2\alpha\text{Fe}_p^{2+} + k_3\text{Fe}_p^{3+}}.$$

If step 4, which is the back reaction regenerating Mb, predominates, then this equation reduces to

$$-\frac{d\text{Fe}_p^{2+}}{dt} = \frac{4k_1k_2}{k_4} (1-\alpha) (\alpha) \text{Fe}_p^{2+} \quad (8)$$

which is identical in form with eqn. 6.

If MbO₂ can also react like Mb in reaction 2 but less rapidly, i.e.



and this reaction replaces reaction 2 at high oxygen pressures, then eqn. 8 becomes

$$-\frac{d\text{Fe}_p^{2+}}{dt} = \frac{4k_1k'_2}{k_4} (1-\alpha) (1-\alpha) \text{Fe}_p^{2+}, \quad (9)$$

which is identical with Eqn. 7.

Although this mechanism can account entirely for the kinetic behaviour obtained from spectrophotometric measurements of the metmyoglobin formation, it leaves unexplained the abnormal consumption of oxygen during the reaction. In a previous paper (George & Stratmann, 1952) it was shown that 2.5 ± 0.3 moles of oxygen are used for each mole of metmyoglobin formed. The stoichiometry in the above mechanism requires the ratio to be 0.25 or 0.5 if the peroxide is utilized in a secondary oxidation. Without detailed knowledge of which groups on the protein molecule are involved no further progress can be made in establishing the precise mechanism. Even though the simple reaction scheme leading to eqns. 8 and 9 is thus inadequate in detail there is good reason to believe it to be correct in principle in so far as the kinetics derive from competition reactions, for such reactions account readily for the form of the rate equation.

SUMMARY

1. The oxidation of myoglobin to metmyoglobin by molecular oxygen at 30° in 0.6M-phosphate buffer, pH 5.69, is shown to be first order in unoxidized myoglobin over a range of oxygen pressures from 0.3 to 760 mm.

2. The observed first order rate constant at first increases with increasing oxygen pressures, shows a well defined maximum value at 1–1.4 mm. partial pressure of oxygen and then decreases to a constant value above 30 mm.

3. The determination of the equilibrium constant for the myoglobin-oxygen reaction under the conditions of the oxidation experiments at 30° gave $K_e = 0.88 \pm 0.12 \text{ mm.}^{-1}$. Hence the partial pressure for half saturation is 1.131 mm. and thus k_{obs} has its maximum value at half saturation.

4. Using this value of K_e the variation of

k_{obs} with oxygen pressure is shown to be of the form

$$k_{\text{obs.}} = 2.62\alpha(1-\alpha) + 0.30(1-\alpha)^2, \quad (a)$$

$$\text{or } k_{\text{obs.}} = 2.30\alpha(1-\alpha) + 0.30(1-\alpha), \quad (b)$$

where α and $(1-\alpha)$ are the fractions of Mb and MbO₂ respectively.

5. The autoxidation of haemoglobin shows the same kinetic characteristics and Brooks's (1935) data can be represented by the equations:

$$k_{\text{obs.}} = 0.68\alpha(1-\alpha) + 0.04(1-\alpha)^2 \quad (a)$$

$$\text{or } k_{\text{obs.}} = 0.64\alpha(1-\alpha) + 0.04(1-\alpha). \quad (b)$$

6. The activation energy at low oxygen pressures (4 mm.) is $19 \pm 1 \text{ kg.cal.}$ and at high oxygen pressures (760 mm.) is $25 \pm 1 \text{ kg.cal.}$ At these extremes the operative form of the equation for $k_{\text{obs.}}$ involves only the first and only the last term respectively.

7. Neither the present data for myoglobin nor Brooks's data for haemoglobin permit a choice between equations *a* and *b* but a consideration of the chemical mechanism including the different activation energies at the extremes of oxygen pressure favour equation *a*.

8. A free radical mechanism for the autoxidation is discussed which involves the participation of an auxiliary electron-accepting group on the protein molecule acting as a catalyst in a reaction regenerating the unoxidized haemoprotein and thus serving to 'protect' the haem from oxidation. However, this mechanism is not complete for it does not account for the additional consumption of oxygen above that required for oxidizing the haem group (George & Stratmann, 1952).

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Studies on Uridine-Diphosphate-Glucose

By A. C. PALADINI AND L. F. LELOIR

Instituto de Investigaciones Bioquímicas, Fundación Campomar, J. Alvarez 1719, Buenos Aires, Argentina

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A previous paper (Caputto, Leloir, Cardini & Paladini, 1950) reported the isolation of the co-enzyme of the galactose-1-phosphate \rightarrow glucose-1-phosphate transformation, and presented a tentative structure for the substance. This paper deals with: (a) studies by paper chromatography of purified preparations of uridine-diphosphate-glucose (UDPG); (b) the identification of uridine-5'-phosphate as a product of hydrolysis; (c) studies on the alkaline degradation of UDPG, and (d) a substance similar to UDPG which will be referred to as UDPX.

UDPG preparations studied by chromatography. Paper chromatography with appropriate solvents has shown that some of the purest preparations of UDPG which had been obtained previously contain two other compounds, uridinemonophosphate (UMP) and a substance which appears to have the same constitution as UDPG except that it contains an unidentified component instead of glucose. This substance will be provisionally referred to as UDPX (Fig. 1a).

The three components have been tested for co-enzymic activity in the galactose-1-phosphate \rightarrow glucose-1-phosphate transformation, and it has been confirmed that UDPG is the active substance. For each mole of uridine of UDPG in a sample extracted from the paper the total phosphate was 2.04, the labile phosphate (15 min. in *N*-acid at 100°) 1.04, and the reducing power (calc. as glucose) after hydrolysis (10 min. in 0.01*N*-acid at 100°) 1.03 moles.

When UDPG is hydrolysed at pH 2 during 10 min. at 100° glucose is liberated and, as shown in Fig. 1b, the UDPG and UDPX peaks are replaced by a slow-moving component which is uridinediphosphate.

Fig. 1c shows the results obtained after inactivating UDPG with alkali. Besides uridine phosphate a fast- and/or a slow-moving sugar ester are formed.

Identification of uridine-5'-phosphate. The product obtained by hydrolysing off with acid the glucose and one phosphate group from UDPG was previously (Caputto *et al.* 1950) postulated to be uridine-5'-monophosphate. However, the hydrolysis curves of this compound resembled more those given by Gulland & Smith (1947) for uridine-2'-phosphate. Since then Brown, Haynes & Todd (1950) have

found that the substance supposed to be uridine-2'-phosphate was uridine-5'-phosphate. The hydrolysis product of UDPG has now been compared with a synthetic specimen of uridine-5'-phosphate. Both substances were found to be identical as judged by chromatographic behaviour (Fig. 1) and by the rate

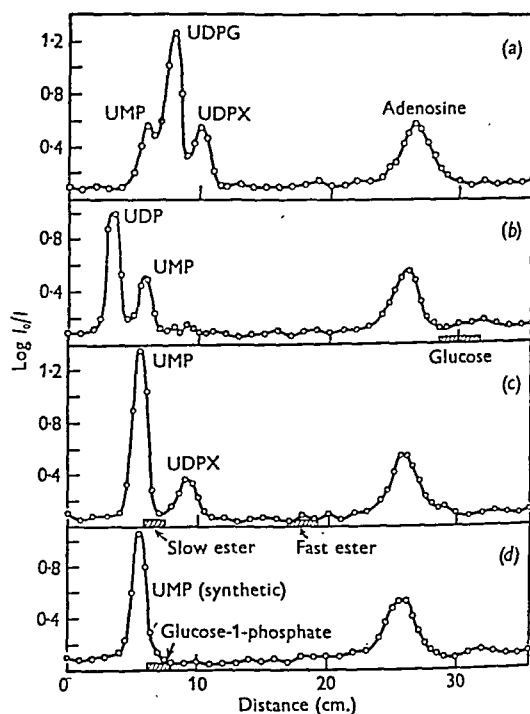


Fig. 1. Chromatograms of UDPG preparations. Samples run simultaneously at 30°. Solvent: ethanol-*N*-ammonium acetate, pH 7.5. Adenosine was added as reference substance. The $\log I_0/I$ values were measured at 260 m μ . a, partially purified UDPG; b, same after heating 15 min. at 100° at pH 2; c, heated 5 min. at 100° with excess NH_4OH ; d, synthetic uridine-5'-phosphate plus glucose-1-phosphate. Glucose and its esters were located after removing the paraffin by ether extraction followed by spraying with aniline phthalate.

of acid hydrolysis (Table 1). The crystalline barium salts of the two substances were prepared, and after recrystallization from water it was found that the microscopic aspect of both samples was the same. The X-ray diffraction patterns obtained by Prof. Galloni were identical for both samples.

Table 1. *Acid hydrolysis of uridine phosphates*(Samples heated at 100° in 0.1 N-H₂SO₄.)

Time (hr.)	P hydrolysed (%)		
	Synthetic uridine-5'- phosphate	UMP from UDPG by acid hydrolysis	UMP from UDPG by alkaline hydrolysis
8.2	12.5	13.7	13.7
20.4	26.5	28.2	29.2
36.5	44.4	46.0	43.9
59.5	57.0	59.7	58.7

The alkaline degradation of uridine-diphosphate-glucose. It has been reported previously (Caputto *et al.* 1950) that UDPG loses its catalytic activity after a mild treatment with alkali. It was found that this inactivation was accompanied by a stabilization of the glucose residue and by the liberation of a secondary acid group of phosphoric acid. Further work on this point has shown that mild alkaline treatment of UDPG leads to the formation of UMP and a glucose ester in which the phosphate is doubly esterified. This substance ('Fast Ester') moves faster than any of the known glucose esters during paper chromatography. With a more drastic alkaline treatment or with acid the 'Fast Ester' is transformed into another substance or substances which move more slowly. These are referred to as 'Slow Ester(s)'.

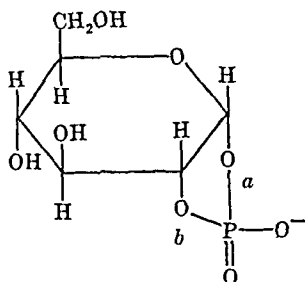


Fig. 2.

The experiments which will be described can be interpreted by assigning to the 'Fast Ester' the structure of a 1:2-monophosphoric ester of glucose (Fig. 2). Further treatment with alkali would yield a mixture of glucose-2- and glucose-1-phosphate by hydrolysis of the links marked *a* and *b* respectively.

Treatment with acid would yield the same products, but since glucose-1-phosphate is hydrolysed immediately only glucose-2-phosphate would remain. Thus the 'Slow Ester' prepared with alkali should be a mixture of glucose-1- and glucose-2-phosphates, while that prepared with acid should be glucose-2-phosphate.

The exact conditions under which UDPG is degraded with alkali have not been determined. It is decomposed rapidly during chromatography with the ethanol-ammonia solvent. Under these conditions the formation of the 'Fast Ester' apparently occurs in less than 20 min., since the latter appears as a well defined spot with practically no tailing. At pH 8 at 18° UDPG remained unchanged during 18 hr. At pH 8.5 in 2 min. at 100° a mixture of UMP, and 'Fast' and 'Slow' ester was formed. In concentrated ammonia at 0° during 30 min. UMP and 'Fast Ester' were formed.

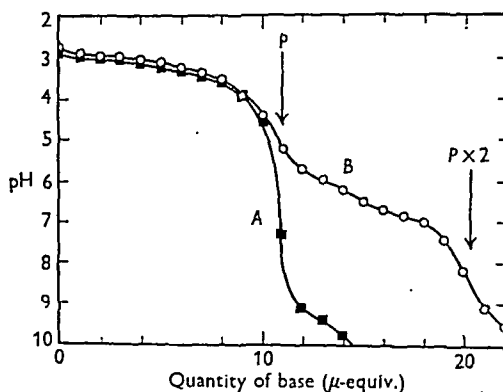


Fig. 3. Titration curve of the 'Fast Ester'. The substance was passed through a column of cation exchange resin in the hydrogen form. One sample was titrated directly (curve A) and another sample (curve B) was heated 15 min. to 100° before titration. After this treatment 10% of inorganic phosphate and 10% of the glucose were liberated. The arrow marked *P* shows the μmoles of phosphate in the sample.

Properties of the 'Fast Ester'. Table 2 shows the *R_F* values of the 'Fast Ester' compared with glucose and glucose-1-phosphate. With the solvents which were used the *R_F* values are grossly inversely proportional to the number of acid groups in the molecule: thus hexosediphosphates move slower than the monophosphates. For the 'Fast Ester' the values

Table 2. *Paper chromatography of the 'Fast' and 'Slow' esters*

(Whatman no. 1 paper.)

Solvent	<i>R_F</i> values			
	'Fast Ester'	'Slow Ester'	Glucose-1- phosphate	Glucose
Ethanol (77% v/v)	0.29	—	0.10	0.42
Ethanol ammonia	0.53	0.17	0.14	0.58
Ethanol ammonium acetate, pH 7.5	0.55	0.22	0.20	0.71

are nearly as high as those of free glucose. This fact was the first indication that the substance contains fewer acid groups than any of the known hexose-monophosphates. This was confirmed by electrometric titration (Fig. 3) which shows the presence of a primary but no secondary acid group. Acid hydrolysis of the 'Fast Ester' yielded a sugar which was identified as glucose by paper chromatography in several solvents. The same result was obtained after hydrolysis with alkaline phosphatase.

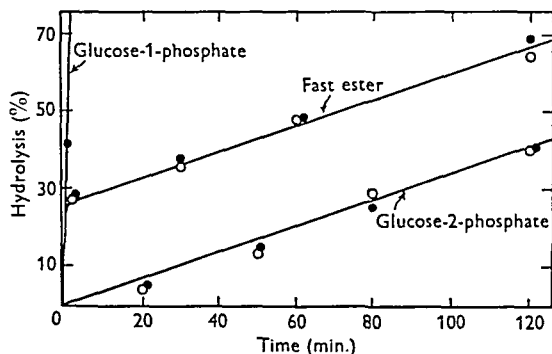


Fig. 4. Acid hydrolysis of the 'Fast Ester'. Samples heated at 100° in 0.1N-H₂SO₄. Reducing power measured with Somogyi's (Somogyi, 1945) copper reagent followed by arsenomolybdic acid (Nelson, 1944). ●—●, phosphate; ○—○, reducing power as glucose.

The curve of hydrolysis of the 'Fast Ester' in 0.1N-acid is shown in Fig. 4. The curve shows a break at about 26% hydrolysis as if it were the curve of a mixture of 26% glucose-1-phosphate and 74% glucose-2-phosphate. The curve of liberation of reducing power is parallel to that of phosphate liberation. With the reagent used (Somogyi, 1945) glucose-2-phosphate does not give a detectable reduction. On heating the 'Fast Ester' in 0.1N-alkali (Fig. 5) 26% glucose-1-phosphate is formed in less than 5 min. and may be detected with the specific phosphoglucumutase test. The hydrolysis curve of the remaining 74% of organic phosphate is similar to that of glucose-2-phosphate.

Heating the 'Fast Ester' in dilute acid for a few minutes leads to the liberation of a secondary acid group of phosphoric acid (Fig. 3). The change can also be detected by paper chromatography, since the 'Fast Ester' is transformed into esters having about the same R_F as the normal hexosephosphates (Table 2).

Osazone formation from 'Slow Ester'. The phosphate liberated during osazone formation was estimated on a sample of 'Slow Ester' obtained from the 'Fast Ester' by heating at 100° for 5 min. in 0.1N-acid. The inorganic phosphate formed by the acid treatment was measured and subtracted from the value obtained after phenylhydrazine treatment. For comparison glucose-1-phosphate and glucose-2-phosphate were also tested. The results were as

follows (% liberation of P): glucose-1-phosphate, 0; glucose-2-phosphate, 94%; 'Slow Ester', 100%.

Liberation of phosphate during osazone formation would appear in theory to be specific for sugars with a free carbonyl containing phosphate in the 1 or 2 positions. However, it has been observed that glucose-3-phosphate (Raymond & Levene, 1929) and fructose-3-phosphate (Levene, Raymond & Walti, 1929) also lose phosphate during osazone formation.

The structure proposed in Fig. 2 for the 'Fast Ester' is consistent with experiments described in a previous paper (Leloir, 1951), in which a 'Fast

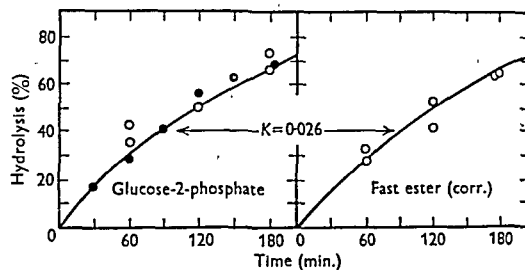


Fig. 5. Alkaline hydrolysis of the 'Fast Ester'. Inorganic phosphate was estimated after the samples (1.1 μ M) were heated in 2 ml. of 0.1N-NaOH to 100° in stoppered bronze tubes. In a parallel experiment glucose-1-phosphate was estimated with yeast phosphoglucumutase (Cardini, Paladini, Caputto, Leloir & Trucco, 1949) activated with glucose diphosphate. Glucose-1-phosphate standards were run at the same time. The samples of the 'Fast Ester' gave 26% of glucose-1-phosphate after heating 5 min. in 0.1N-NaOH, and the values remained constant after heating 10 or 20 min. Glucose-1-phosphate was not affected by phosphoglucumutase. The corrected values for the percentage hydrolysis of the 'Fast Ester' were calculated by considering the total phosphate minus the glucose-1-phosphate as equal to 100. ●, Farrar's data; ○, this paper.

'Ester' containing galactose was detected besides that containing glucose. Evidence for the α structure of glucose in UPG has been obtained from preliminary polarimetric observations. It was found that an acid treatment which hydrolysed off the glucose produced a decrease in dextrorotation: $\Delta[M] = 183^\circ$. This value is similar to that for the conversion of α -glucose-1-phosphate to α - β -glucose ($\Delta[M] = 218^\circ$). For an α -glucose ester the likely positions for the formation of a cyclic phosphate would be 1:2 or 1:4. But since 1:4 would be very unlikely for an α -galactose ester it was concluded that both the glucose and the galactose esters were probably esterified at the 1 and 2 positions.

It may be mentioned that Forrest & Todd (1950) have described the formation of a cyclic phosphate of riboflavin by alkaline treatment of flavin-adenine-dinucleotide. Periodate oxidation used 1 mole of oxidant and gave no formic acid so that it was concluded that the phosphate was esterified at positions 4 and 5 of the ribityl residue.

UDPX. The substance giving the small peak which runs faster than UDPG (Fig. 1a) has been isolated in small amounts by paper chromatography. Analysis showed that the ultraviolet spectrum at different pH values and after bromine treatment was that of uridine, and that for each mole of uridine in UDPX the total phosphate was 2.04, labile phosphate (15 min. in N-acid at 100°) 1.0, and reducing power after hydrolysis (10 min. in 0.01N-acid at 100°, calc. as glucose) 0.5 mole.

UDPX was found to remain unaffected by a treatment with alkali sufficient to decompose UDPG (Fig. 1c).

The unknown component of UDPX has been studied by chromatography in various solvents, and it has been found to be different from the following substances: aldohexoses, pentoses, fructose, tagatose, sorbose, glucosamine, uronic acids, fucose, rhamnose, xylulose, ribulose, deoxyribose, adonose, erythrulose, 1- and 3-methyl fructose, 2- and 3-methyl glucose, glyceraldehyde and dihydroxyacetone.

UDPX was found to be clearly different from UDP galactose (Leloir, 1951) and from the compound found by Park & Johnson (1949) and Park (1950) in *Staphylococcus aureus*. The R_F values, both of the intact substances and of the sugars obtained by hydrolysis, were different.

The substance X was found to be unfermentable by baker's yeast and to give negative results in the following tests: resorcinol for ketoses (Roe, 1934); Elson & Morgan (1933) for amino sugars, orcinol for pentoses (Mejbaum, 1939), and the test for methyl pentoses (Nicolet & Shinn, 1941). With the aniline-phthalate reagent it gave a brownish-yellow colour which only appeared after prolonged heating.

EXPERIMENTAL

Methods. Analytical methods and preparations were as described in previous papers (Caputto *et al.* 1950). Glucose-2-phosphate was prepared from diphenyl 1:3:4:6-tetraacetyl- β -D-glucose-2-phosphate (Farrar, 1949) kindly supplied by Mrs K. R. Farrar. A sample of synthetic uridine-5'-phosphate was obtained from Prof. A. R. Todd.

The 'Slow Ester' was usually prepared by paper chromatography of UDPG with ethanol-ammonia as solvent. The position of the substance was revealed in a small part of the paper with aniline phthalate and the ester was subsequently extracted with water.

Paper chromatography. Descending chromatography was usually employed, except when the experiments were carried out in a thermostat. In these cases a more compact chamber similar to that described by Block (1950) was used in which the solvent travels first upwards and then downwards.

Whatman paper no. 1 was employed throughout. For nucleotides and phosphoric esters it was found convenient to wash the paper with 2N-acetic acid (Hanes & Isherwood, 1949). Usually a pad of blotting paper was stapled at the

end of the strips, and the position of the substances was referred to appropriate substances such as glucose for sugars and adenosine for nucleotides. The solvents used were: (a) 7.5 vol. of 95% ethanol plus 3 vol. of M-ammonium

Table 3. $R_{\text{adenosine}}$ values, at $20 \pm 1.5^\circ$ of some purines, pyrimidines and derivatives

Substance	Solvent	
	Ethanol-ammonium acetate, pH 7.5	Ethanol-ammonium acetate, pH 3.8
Thymine	—	1.25
Uracil	1.13	1.16
Uridine	1.13	1.16
Cytidine	—	1.10
Adenine	1.06	1.00
Hypoxanthine	—	1.00
Adenosine	1.00	1.00
Uridine-3'-phosphate	0.46	0.94
Guanosine	—	0.88
Cytidylic acid	0.34	0.85
Uridine-5'-phosphate	0.35	0.84
Adenosine-3'-phosphate	0.29	0.76
Adenosine-5'-phosphate	—	0.73
UDPX	0.55	0.73
UDPG	0.43	0.65
Guanylic acid	0.22	0.63
Uridine diphosphate	0.14	0.59
Diphosphopyridinenucleotide	0.21	0.32
Adenosinetriphosphate	0.07	—
Xanthine	0.0	0.0
Guanine	0.0	0.0

acetate (pH ~ 7.5); (b) same as (a) but with M-ammonium acetate buffer of pH 3.8; and (c) 7.5 vol. of 95% ethanol plus 3 vol. of concentrated ammonia. With solvent (a) the nucleotides give values of $R_{\text{adenosine}}$ below 0.7, while the nucleosides give higher values (Table 3). The R_F values of nucleotides vary with the pH of the solvent (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) and with temperature. As shown in Fig. 6, the changes with temperature are not parallel for all the substances.

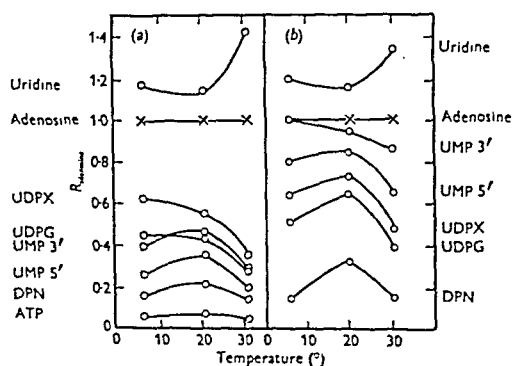


Fig. 6. Paper chromatography of some purine and pyrimidine derivatives. Ethanol-ammonium acetate solvents. a, of pH 7.5, and b, of pH 3.8, as described in text.

The position of ultraviolet-absorbing substances was ascertained by measuring the extinction at 260 m μ . after impregnation of the paper with liquid paraffin. A standard Beckmann spectrophotometer was used with an accessory

which allowed strips of paper to be run along the photocell entrance (Leloir & Paladini, 1951).

Sugars and their esters were revealed with aniline phthalate reagent (Partridge, 1949).

Liberation of phosphate with phenylhydrazine. This procedure was described by Deuticke & Hollmann (1939) for the estimation of fructosediphosphate. The analytical procedure has been modified by Dr Cardini as follows:

Reagents: (a) 6% (w/v) phenylhydrazine hydrochloride in water (decolorized with charcoal if necessary); (b) saturated solution of sodium acetate; (c) saturated Na_2SO_3 .

The samples and phosphate standards in 0.5 ml. of water plus 0.1 ml. of (a), 0.05 ml. of (b) and 0.1 ml. of (c) were heated 30 min. in a boiling-water bath. After cooling 0.75 ml. of 5N- H_2SO_4 , 0.75 ml. of 2.5% ammonium molybdate and water to a total vol. 7.5 ml. were added. After 10 min. the optical density was measured at 660 m μ . Controls heated without phenylhydrazine were run at the same time.

SUMMARY

1. Purified preparations of uridine-diphosphate-glucose (UDPG) were studied by paper chromato-

graphy and found to be contaminated with uridylic acid and a substance UDPX.

2. The uridylic acid obtained by degradation of UDPG has been identified as uridine-5'-phosphate.

3. The alkaline degradation products of UDPG are uridine-5'-phosphate and a cyclic phosphate ester of glucose, probably esterified at positions 1 and 2 of the glucose. This ester decomposes with acid or alkali giving glucose-1-phosphate (25%) and glucose-2-phosphate (75%).

4. The contaminating substance UDPX appears to have the same structure as UDPG except that it contains an unidentified component instead of glucose.

The studies with synthetic uridine-5'-phosphate and with many samples of rare sugars were possible owing to the kindness of Prof. A. R. Todd, F.R.S., and the identification of glucose-2-phosphate by the generosity of Mrs K. R. Farrar. We wish to express our thanks to them as well as to Prof. E. E. Galloni for the X-ray diffraction studies, to Dr C. E. Cardini for his co-operation with the phenylhydrazine method and to Dr J. T. Park for a sample of the *Staphylococcus aureus* compound.

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The Structure of Urorosein

By J. HARLEY-MASON AND J. D. BU'LOCK
Chemical Laboratory, University of Cambridge

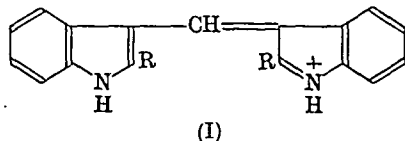
(Received 19 November 1951)

Urorosein is a red pigment obtained by the action of mineral acids on the tryptophan oxidation product indole-3-aldehyde (Ellinger & Flamand, 1909). Fearon & Boggust (1950) have reviewed earlier work on the pigment and assigned to it the structure dehydro-indolo-3':2':2:3-carbazole. This is the correct systematic name for the structure as shown

by Fearon & Boggust and described by them as indolo-3':2':2:3-carbazole. It is, however, difficult to see how such a structure containing only a *p*-quinone-diimine chromophore could be so intensely coloured and, moreover, the absorption spectrum as quoted shows a close correspondence to those of the di-3-indolylmethenes described by König (1925). It

seemed desirable, therefore, to re-investigate the structure of this pigment. Fearon & Boggust prepared urorosein sulphate to which they assigned the formula $(C_{18}H_{10}N_2SO_4)_3 \cdot H_2SO_4$ and further stated that no evidence could be obtained of the splitting off of an aldehyde side chain in the course of formation of the pigment from two molecules of indole-3-aldehyde.

We have repeated the preparation of the sulphate and have made, in addition, the chloride, bromide and perchlorate. Analyses of these salts showed that they are in fact derived from a base of formula $C_{17}H_{12}N$, and it is thus clear that one carbon atom is



lost in the course of the condensation. We have further shown that this carbon atom is in fact lost as formic acid, which was identified in a distillate of the mother liquors from the preparation of the pigment. Ellinger & Flamand (1909) had earlier shown that 2-methylindole-3-aldehyde was converted by acids into 2:2'-dimethyl-di-3-indolylmethene salts (I; $R = Me$) with the elimination of formic acid, so that it appeared from our results that indole-3-aldehyde reacted in an exactly similar manner to give di-3-indolylmethene salts (I; $R = H$). A synthesis of such salts by the reaction of indole with ethyl orthoformate followed by treatment with acids was described by König (1911). We repeated this work and prepared di-3-indolylmethene chloride, bromide, sulphate and perchlorate. These salts were identical in every way with the urorosein salts obtained from indole-3-aldehyde. Melting points were found to be unsatisfactory criteria of identity, since salts all decompose rather indefinitely at temperatures varying with the rate of heating. Identity was therefore established by X-ray powder photographs and absorption spectra. The statement of König (1911), that the sulphate obtained by the orthoformate method and Ellinger & Flamand's urorosein sulphate are spectroscopically identical, is thus confirmed. As Fearon & Boggust observe, the salts tend to dissociate in dilute solution and the absorption spectra were therefore determined in the presence of free acid. The intense colour of the salt is of course due to resonance, the positive charge being shared between the two nitrogen atoms in two equivalent mesomeric structures.

By reaction of ethyl orthoformate for a longer period with excess of indole, a colourless product was obtained; this was tri-3-indolylmethane, which on heating with acids was converted into di-3-indolylmethene salts with elimination of a molecule

of indole. Tri-3-(2-methylindolyl)methane has been shown to behave similarly.

In agreement with Fearon & Boggust, we have found that indole-3-acetic acid yields a red pigment, apparently urorosein, on warming with acids in the presence of oxidizing agents, but we have been unable to confirm their statement that a similar pigment is obtained from 2-methylindole.

EXPERIMENTAL

Preparation of urorosein salts

Preparation from indole-3-aldehyde. Finely powdered indole-3-aldehyde (0.5 g.) was heated at 70° for 1 hr. with 35% (w/v) $HClO_4$ (400 ml.) with stirring. After standing overnight the product, which had crystallized partially during the heating, was collected and recrystallized from acetic acid. Urorosein perchlorate formed deep-red needles with an intense green reflex and containing a molecule of acetic acid of crystallization. (Found: C, 56.3; H, 4.0; N, 7.0. $C_{17}H_{13}N_2ClO_4 \cdot C_2H_4O_2$ requires C, 56.8; H, 4.2; N, 6.95%.)

Other salts. Urorosein chloride. This was obtained in the form of deep-red needles from ethanol-acetone. (Found: C, 72.3; H, 4.9; Cl, 12.4. $C_{17}H_{13}N_2Cl$ requires C, 72.7; H, 4.6; Cl, 12.6%.)

The bromide crystallized in the form of deep-red prisms from ethanol. (Found: C, 62.4; H, 4.3; Br, 24.6. $C_{17}H_{13}N_2Br$ requires C, 62.8; H, 4.0; Br, 24.5%.) Urorosein sulphate formed deep-red plates from acetic acid. (Found: C, 60.1; H, 4.0; N, 8.0. $C_{17}H_{13}N_2 \cdot HSO_4$ requires C, 59.8; H, 4.1; N, 8.2%.) These salts were prepared exactly similarly to the perchlorate using 20% (w/v) HCl , 20% (w/v) HBr and 30% (w/v) H_2SO_4 .

Preparation from indole and ethyl orthoformate. Indole (1.2 g.) and ethyl orthoformate (0.8 g.) were dissolved in ethanol (5 ml.), 1 drop of dilute ethanolic HCl added as catalyst and the mixture refluxed for 1 hr. After cooling, the deep-yellow solution was diluted with ethanol (50 ml.) and then 20% (w/v) $HClO_4$ (50 ml.) added with stirring. Urorosein perchlorate crystallized out and was collected and recrystallized as above. The sulphate, chloride and bromide were prepared similarly using the appropriate acids in place of $HClO_4$.

The ultraviolet absorption spectra, determined with a Beckmann spectrophotometer, of the bromides and perchlorates prepared by the two methods above in 95% ethanol containing 5% HBr and $HClO_4$ respectively were compared and found to be identical, having maxima at 258, 286, 487 and 530 $m\mu$. X-ray powder photographs of the bromides prepared by the two methods were identical.

The action of NH_3 on urorosein salts gave the yellow free base in crystalline form: the material was, however, unstable and could not be obtained analytically pure.

Tri-3-indolylmethane

Indole (2 g.) and ethyl orthoformate (0.8 g.) were dissolved in ethanol (7 ml.) one drop of ethanolic HCl added and the mixture refluxed for 6 hr. Towards the end of the heating some pale-yellow crystalline material began to separate. After cooling the solid was filtered off and recrystallized from ethanol. Tri-3-indolylmethane formed

colourless prisms, m.p. 244–246°. (Found on a sample dried at 100°/10 mm.: C, 83.2; H, 5.0; N, 11.6. $C_{25}H_{19}N_3$ requires C, 83.0; H, 5.25; N, 11.6%.)

Detection of formic acid

Indole-3-aldehyde (0.2 g.) was treated with 10% (w/v) HCl (100 ml.) at 60° for 20 min. and the urorosein chloride filtered off. The mother liquor was distilled at atmospheric pressure and formic acid was detected in the first portion of

the distillate by reduction to formaldehyde with Mg and reaction with chromotropic acid (Feigl, 1946).

SUMMARY

Urorosein salts are shown to be derived from di-3-indolylmethene and can be prepared either from indole-3-aldehyde or from indole and ethyl orthoformate.

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The Fractionation and Enzymic Breakdown of some Phosphorus Compounds in Leaf Tissue

By MARGARET HOLDEN

Rothamsted Experimental Station, Harpenden, Herts

(Received 28 September 1951)

In a recent paper Pirie (1950) has described the preparation and properties of a nucleoprotein, from the sap of healthy tobacco leaves, which has some similarity to plant viruses. The present paper is concerned with the fractionation of the phosphorus of leaf tissue to find how much of the phosphorus of the leaf is in the form of nucleic acid. The enzymic breakdown of some of the phosphorus-containing compounds has also been studied.

Although there is an extensive literature on the nature and fractionation of phosphorus compounds in animal tissues, plant tissues have received less attention. Until recently, work on plant tissues has been mainly confined to seeds. When leaves have been used the material has usually been dried as a preliminary. This will vitiate some of the results, for there would be changes due to enzyme action during drying, especially at room temperature. Knowles & Watkin (1932) investigated the phosphorus compounds in wheat at different stages of growth. They used air-dry material, which probably accounts for the very high figures for inorganic phosphorus. They claim to have found phytin in vegetative parts of the wheat plant, but other workers have been unable to find any phytin except in seeds. DeTurk, Holbert & Howk (1933) studied the chemical transformations of phosphorus in the growing corn plant and found that phytin disappeared rapidly on germination. They found differences in the phosphorus distribution between fractions when the material was slowly and rapidly dried.

Heard (1945) investigated the forms of phosphorus in a trichloroacetic acid extract of fresh barley seedlings. In a study of the nucleic acid content of green leaves, von Euler & Hahn (1947, 1948) extracted ribonucleic acid with sodium hydroxide as in the Schmidt & Thannhauser (1945) procedure for animal tissues. The nucleic acid was precipitated as the lanthanum salt and the pentose content determined by a phloroglucinol method. Pectic substances in the extract were precipitated with the nucleic acid, and estimation of the nucleic acid content based on the pentose figure gave values which were too high. Williams (1945) described a method for estimating nucleic acid phosphorus in plant material in which the dry fat-free tissue was heated with 12.5% (w/v) sodium chloride in the

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presence of triacetin. The nucleic acid in the extract was then precipitated at pH 1. In this method no distinction was made between ribo- and deoxyribonucleic acids. The method of Ogur & Rosen (1950) which, with modifications, was used in the present investigation for the differential extraction of ribo- and deoxyribo-nucleic acids was used by them on fresh corn root tips.

The enzymes in plant tissues concerned with the breakdown of phosphorus compounds have also been studied much less frequently than those of animal tissues. The presence of phosphatase activity in leaves is well known. Courtois & Khorsand (1950) have recently found that there are two phosphatases, one with an optimum pH at 5.0–5.2 and the other at about pH 4. Schlamowitz & Garner (1946) recognized a ribonuclease ('ribonucleinase') in sprouted soy beans. Pirie (1950) found that tobacco-leaf sap had ribonuclease activity. Hanahan & Chaikoff (1948) found a phospholipin-splitting enzyme in cabbage leaves which was able to attack only the nitrogenous base-phosphoric acid linkage of lecithin with the formation of choline and phosphatidic acid. Ducet (1949) found this enzyme and also lecithinase B, which splits lecithin to glycerophosphorylcholine and fatty acids, in numerous other plants such as potato, bean, pea and ryegrass.

MATERIAL AND METHODS

Leaves from glasshouse-grown tobacco plants (*Nicotiana tabacum* var. White Burley) were used for most of the work. Other species used are mentioned in the text. The midribs were cut out, the laminae minced in a domestic meat mincer and the sap squeezed out by hand through madapollam into a cooled receiver. The residue in the cloth, called 'fibre', was washed three times by suspending in a volume of distilled water about equal to that of the sap and squeezing out. If not used at once the fibre was stored at 4° with CHCl₃ added. The sap was centrifuged at 8000 rev./min. (6000g) for 15 min. and the deposit obtained called the 'chloroplast fraction'. This was washed twice by suspending in distilled water and recentrifuging at 8000 rev./min., then it was finally suspended in water and stored in the refrigerator.

For some experiments much of the starch was removed from the chloroplast fraction by scraping off the upper green layer from the white starch layer in the deposit obtained on centrifuging. The green material was suspended in water, centrifuged down again and separated from the starch.

This was repeated until there was no obvious starch layer.

Phosphorus. This was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic P were obtained by developing the colour in samples without incineration.

Dry matter of fibre and of aqueous fractions. This was determined by drying samples in an oven at 100° overnight. Ethanol-ether fractions were dried over H₂SO₄ in a vacuum desiccator.

Nitrogen. Total N was determined by a micro-Kjeldahl method using SeO₂:CuSO₄:K₂SO₄ (1:1:8) catalyst.

Carbohydrate. Total carbohydrate was determined by the orcinol method (Pirie, 1936), reducing sugar by the Hanes (1929) modification of the Hagedorn & Jensen method and uronic acid by the method of Tracey (1948).

Deoxyribonucleic acid (DNA). This was determined by the diphenylamine method (Dische, 1930) and the cysteine-sulphuric acid method (Stumpf, 1947), and a modification of the tryptophan-perchloric acid method of Cohen (1944).

Determination of phosphatase activity. The liberation of inorganic P from sodium β -glycerophosphate was used to measure phosphatase activity. For the tests a total volume

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph and with a Unicam quartz spectrophotometer.

pH measurements. These were made with a glass electrode.

RESULTS

FRACTIONATION OF PHOSPHORUS COMPOUNDS IN FIBRE, CHLOROPLAST AND SOME SAP FRACTIONS

Ogur & Rosen (1950) have recently described a method of fractionating P compounds in plant tissue involving the use of perchloric acid. This method has been modified, and forms the basis of the fractionation procedure used in the work described here.

Fibre

The P compounds of tobacco-leaf fibre have been fractionated into four groups: (1) P soluble in cold dilute acid; (2) P soluble in ethanol-ether; (3) P soluble in *n*-HClO₄ on soaking overnight at room temperature; (4) P soluble in *n*-HClO₄ on incubating at 37° overnight. The extraction of these removed over 98% of the fibre P. Table 1 shows the results of a typical experiment.

Table 1. *Fractionation of phosphorus in fresh and incubated fibre*

(3 g. lots of fibre (dry matter 0.617 g.) extracted successively as shown in the table. The method is described in the text, p. 434. The water extract from the incubated fibre was obtained by incubating the fibre twice in water at 37° overnight.)

Extractant	P in fresh fibre		P in incubated fibre	
	(mg./g. of dry fibre)	(% of total in fibre)	(mg./g. of dry fibre)	(% of total in fibre)
1. Water, 30 ml.	0.097	3.3	2.356	76.1
2. 0.2N-HClO ₄ , 30 ml.	0.102	3.5	0.073	2.4
3. Ethanol-ether (3:1), 40 ml., i.e. lipid I	0.64	21.8	0.082	2.6
4. <i>n</i> -HClO ₄ at 16° overnight, 30 ml., i.e. RNAP*	1.61	54.8	0.194	6.3
5. Two extractions with <i>n</i> -HClO ₄ at 37° overnight, 40 ml., i.e. mainly DNAP†	0.485	16.5	0.391	12.6
Totals	2.934	—	3.096	—

* RNAP, ribonucleic acid phosphorus.

† DNAP, deoxyribonucleic acid phosphorus.

of 5 ml. contained sodium β -glycerophosphate solution (pH 6) to give 100 μ g. P/ml., sodium citrate buffer (pH 6) 0.04M, enzyme solution and water to make up the volume. Samples were removed at intervals and pipetted into 5 ml. of 2N-H₂SO₄ and inorganic P determined.

Determination of nuclease activity. The liberation of P not precipitable by uranyl nitrate in trichloroacetic acid (TCA) (MacFadyen, 1934) from yeast nucleic acid was used to measure ribonuclease activity. For the tests a total volume of 5 ml. contained sufficient of the sodium salt of yeast ribonucleic acid to give 100 μ g. P/ml., sodium citrate buffer 0.04M (pH 6), enzyme solution and water to make up the volume. Samples were removed at intervals, made up to 1 ml., and 1 ml. uranyl nitrate solution 0.5% (w/v) in 2.5% (w/v) TCA was added. After standing the tubes on ice for 10 min. the precipitates were centrifuged down and total P determined in the supernatants. Deoxyribonuclease activity was determined similarly, using a solution of the sodium salt of deoxyribonucleic acid from thymus (British Drug Houses Ltd.) and either sodium diethylbarbiturate-sodium acetate buffer (pH 7), or an α -picoline-acetic acid buffer (pH 6) (Cecil, 1950).

As soon as possible after mincing and washing, two 3 g. lots of fibre were suspended in 20 ml. water. One lot was incubated twice, each time for about 24 hr. at 37° with CHCl₃ present. The other was squeezed out at once and re-washed with 10 ml. water. The fibre was then extracted with two lots of 0.2N-HClO₄, 20 ml. and 10 ml., which were combined. The acid was not left in contact with the fibre for longer than 10 min. at room temperature. The fibre was then extracted with ethanol-ether (3:1) at room temperature until no more colour could be removed, as the removal of P paralleled the removal of pigments. The fibre was then suspended in 20 ml. *n*-HClO₄ and kept at room temperature overnight, i.e. about 16° for 18 hr. After removal of the extract the fibre was washed with 10 ml. *n*-HClO₄ and the *n*-HClO₄ extracts pooled. The fibre was then suspended in 20 ml. *n*-HClO₄ and incubated at 37° overnight, washed with 10 ml. *n*-HClO₄ and the extracts pooled. The extract from a second incubation at 37° overnight with 10 ml. *n*-HClO₄ was added to the first incubation extract.

The portion which was incubated at 37° in water was afterwards extracted with ethanol-ether and HClO₄ in exactly the same way as the other portion.

(1) *Cold, dilute acid-soluble P.* For the extraction of this fraction 0.2N-HCl, 0.2N-HClO₄ and 5% (w/v) TCA were used and the results compared. There was no significant difference in the amount of P extracted by these acids. This fraction amounted to not more than 10% of the total P in well washed fresh fibre. From 30 to 50% of the P in this fraction was not in the form of PO₄— and its nature has not been investigated. In fibre that had been stored in the refrigerator for 2–3 weeks as much as 30% of the total P of the fibre was in the acid-soluble fraction. The increased amount extractable was inorganic P due to enzymic breakdown of P compounds and could be removed with water alone, without acid.

(The small leaves were less than 10 cm. long and the large from 10 to 25 cm. long.) This shows that a much smaller proportion of the N was lost on incubation than of the P. The phospholipid-splitting enzyme investigated by Hanahan & Chaikoff (1948) caused only 5% loss of ether-soluble P compared with 35% loss of N when *soy bean* phospholipin was used as substrate. In the present investigation when cabbage-leaf fibre was incubated in water at 37° it behaved similarly to tobacco with the ethanol-ether soluble P being much reduced.

(3) and (4) *P soluble in N-HClO₄.* When fibre which had been washed with cold 5% (w/v) TCA was heated at 90° for 15 min. in 5% TCA (Schneider, 1945) or incubated at 37° for

Table 2. *Extraction of phospholipid from fibre*

(10 g. fibre (1.98 g. dry matter, 6.13 mg. P) extracted successively as shown in the table.)

Extractant	Dry matter		P		
	(mg.)	(% total in fibre)	(mg.)	(% total in fibre)	(mg./g. dry matter of fraction)
1. Ethanol-ether, 144 ml.	279	14.1	0.216	3.5	0.8
2. 2% (w/v) TCA, 84 ml.	—	—	0.337	5.4	—
3. Ethanol-ether, 98 ml.	85	4.3	1.120	18.1	13.2

Table 3. *Composition of ethanol-ether extracts from fresh and incubated fibre of large and small leaves*

(Two lots (10 g.) of fibre (3.20 g. dry matter, 7.5 mg. P) from large leaves (10–25 cm. long) and 2 lots (10 g.) of fibre (2.35 g. dry matter, 10.9 mg. P) from small leaves (<10 cm. long). The fresh fibres were extracted with 70 ml. 0.2N-HClO₄ and then with 120 ml. ethanol-ether. The incubated fibres were first incubated at 37° overnight in water, then extracted with 0.2N-HClO₄ and ethanol-ether in the same way as the fresh fibres.)

	Large leaves		Small leaves	
	Fresh	Incubated	Fresh	Incubated
Phosphorus:				
mg./g. dry matter of fibre	0.41	0.11	0.70	0.15
% total in fibre	17.4	4.8	15.1	3.3
Nitrogen:				
mg./g. dry matter of fibre	1.8	1.3	2.4	1.4
% total in fibre	3.9	2.8	4.2	2.5
Dry matter (% total in fibre)	15.3	12.4	19.8	14.4

(2) *Ethanol-ether soluble P.* Most of the pigments and about 80% of the lipid material were removed from fibre by extraction with neutral ethanol-ether at room temperature, or with hot solvents in a Soxhlet extractor, without removing more than a small fraction of the phospholipins. After extraction of the fibre with acid, i.e. below pH 2, 15–35% of the fibre P became soluble in cold ethanol-ether. Subsequent extraction with hot solvents showed that extraction in the cold was effective in removing lipid P. A phospholipin fraction with a P content of about 1.5% was obtained by first extracting the fibre with neutral ethanol-ether, then with dilute acid and again with ethanol-ether. Up to 20% of the fibre dry matter was soluble in ethanol-ether. Results are given in Table 2 showing the separation of phospholipin from other lipids.

Incubation of fibre in water decreased the amount of P soluble in ethanol-ether following acid extraction by as much as 90% (Table 1). The dry matter extracted was diminished by less than 20%. Table 3 compares the composition of the ethanol-ether fraction, following acid extraction, from fresh and incubated fibre of large and small leaves.

several hours in acid of the same strength all the P which was brought into solution was in organic form. Lipid P, if not already removed, was extracted in addition to nucleic acid and over 90% of the fibre P was brought out. Ogur & Rosen (1950) found that ribonucleic acid (RNA) could be extracted from root tips, without removing deoxyribonucleic acid (DNA), by soaking in N-HClO₄ at 4° for 18 hr. The DNA was then extracted by heating the residue with two lots of 0.5N-HClO₄ for 20 min. at 70°. They used HClO₄ instead of TCA which had been used for much of the earlier work on P fractionation in animal tissues, because unlike TCA it has almost negligible absorption in the ultraviolet. With leaf tissues the RNA is not extracted so easily, and either a higher temperature or a higher concentration of acid has to be used. To remove all the RNA it is necessary to use conditions which will cause some of the DNA to become soluble. Fig. 1 shows the amount of P extracted from acid and ethanol-ether treated fibre with three concentrations of HClO₄ (3, 1.0 and 0.33N) at 4 and 16° and also 1.0N at 37°. DNA was determined in the extracts by the diphenylamine method. The amount of DNA was negligible except in the

3N-HClO₄ extract at 16° after 2 hr. and in all the samples of N-HClO₄ at 37°. For the routine determination of RNA the fibre was extracted with N-HClO₄ at room temperature overnight. However, the amount of RNA extracted under these conditions does not represent the true total. A second extraction with N-HClO₄ at 16° will remove a small amount more RNA but some DNA then becomes soluble. In the chloroplast fraction (p. 438), where results are not complicated by the presence of DNA, it is clear that some of the RNA is not brought out with N-HClO₄ at 16°, but that a higher temperature had to be used to extract it. Much of the RNA of fibre is due to the presence of chloroplasts so the same is to be expected of the fibre RNA.

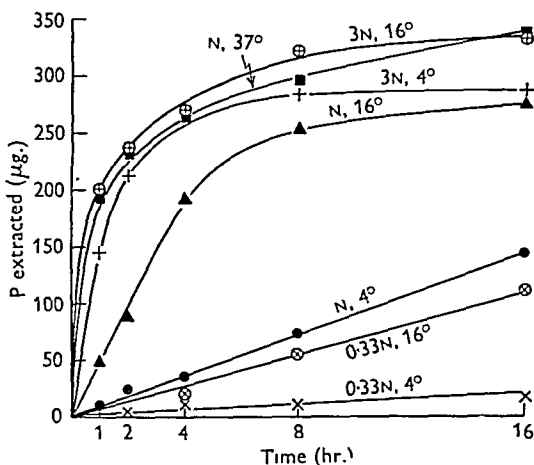


Fig. 1. Extraction of P from fibre with HClO₄. 0.5 g. lots of acid and ethanol-ether extracted fibre (dry matter, 141 mg.; P, 410 µg.) in 5 ml. HClO₄ (0.33N, N, or 3N) for the times and at temperatures shown.

Less than 5% of the total P in extracts made with N-HClO₄ at 16° was inorganic. The carbohydrate content was low, but when 2–3 vol. ethanol were added a precipitate appeared which was mainly 'soluble starch' and pectin. The precipitate contained up to 10% of the P of the extract. When saturated Ba(OH)₂ was added to the ethanolic solution to raise the pH to about 9 a precipitate formed containing 60–80% of the P originally present in the extract. The ultra-violet absorption of a N-HClO₄ extract had a maximum at about 260 mµ. and absorbed more strongly than an aqueous solution of yeast ribonucleic acid of the same P content. Ogur & Rosen (1950) found that treatment of nucleic acid samples with N-HClO₄ increased the absorption at 260 mµ. and this was confirmed using yeast ribonucleic acid.

For extraction of DNA the fibre residue was incubated at 37° with N-HClO₄ overnight. One extraction removed the greater part of the P remaining in the fibre, while a second extraction lowered the P content of the residue to a level where determinations became uncertain. A longer extraction at a lower temperature was used instead of the conditions of Ogur & Rosen. This was to minimize degradation of pectic substances because of the interference of galacturonic acid in colorimetric methods of determining DNA (Holden, unpublished). Determinations of DNA by the diphenylamine and tryptophan-HClO₄ methods usually agreed fairly well. The results obtained by the cysteine-sulphuric acid method were always lower than by the other two methods. The observed P content of the extract was

usually higher than the P to be expected from the colour intensity given by the extract compared with thymus nucleic acid standards of known P content. This is not unexpected for some RNA is present. When agreement between the observed P and the value calculated from deoxyribose determinations was very close it was probably fortuitous, due to interfering substances increasing the absorption at the wavelengths used for colorimetric determinations of deoxyribose.

Extracts made with N-HClO₄ at 37° contained more carbohydrate than those made at room temperature, but this was mainly non-reducing. Much of the carbohydrate was precipitated in a bulky gelatinous form, with up to 20% of the P of the extract in it, when 2–3 vol. ethanol were added. From 50 to 70% of the P originally present in the extract was precipitated on addition of saturated Ba(OH)₂ to the ethanolic solution.

Nitrogen determinations on N-HClO₄ extracts were done after precipitation of KClO₄ at 4°, as the presence of large amounts of HClO₄ is known to cause loss of N during incineration (Weeks & Friminger, 1942). During the fractionation procedure about 25% of the total N of the fibre was removed, of which about 5% was in the ethanol-ether fraction. There was a greater loss of N when fibre was incubated before fractionation, as up to 15% of the fibre N appeared in the incubation extract. With the usual ratio of fibre to acid each N-HClO₄ extract contained about 7% of the fibre N, or 3 mg./g. of the fibre dry matter, whether the fibre had been previously incubated or not.

Incubating fibre in water at 37° diminished the amount of P in the ribonucleic acid fraction by as much as 90%. This is due to the action of ribonuclease present in the fibre (p. 441). There was less diminution in the amount of P in the deoxyribonucleic acid fraction, a loss of from 15 to 35% was found with different batches of fibre. Incubating the fibre in the presence of Mg salts did not cause a greater decrease in the amount of P in this fraction.

Phosphorus fractions in the fibre of leaves of different ages

Pirie (1950) found that the greatest yield of nucleoprotein was from the sap of small young leaves. Table 4 shows the results obtained in one experiment on the fractionation of P in the fibre of leaves of different ages. The leaves were from tall plants, about to flower, and the leaves were grouped according to size and position on the plants. The leaves in the group 'young small' were under 10 cm. long, those in the group 'young large' were 10–20 cm. long and those in the group 'old large' were over 20 cm. long. Some of the leaves in the latter group were slightly yellowed but no completely yellow or withered leaves were included. The total P/g. of the dry matter was highest in the youngest leaves. The acid-soluble P as a percentage of the total P was slightly higher in large leaves than in small but per g. of the dry matter was lower. The inorganic P in this fraction remained fairly constant per g. of the dry matter, but the P in organic form was lower in the large leaves. The phospholipin P/g. of the dry matter was about the same in the different aged leaves, but was a much higher percentage of the total P in the large leaves. Both RNAP and DNAP formed a lower percentage of the total P in the large leaves than in the small and the amount per g. of dry matter was much lower. In small young leaves the RNA content was of the order of 35 mg./g. of the dry matter.

dry matter was very variable, depending partly on the age of the leaf and on how much starch was present. The amount of acid-soluble P was about 10% of the total present which is of the same order as that in fibre. The ethanol-ether soluble P was 25–45% of the total in the fraction, which is a higher range than in fibre. As with fibre, most of the phospholipid P was not extractable until after acid treatment.

Extraction with $N-HClO_4$ at room temperature removed about 70% of the P still present after extraction of lipid P; a higher temperature was needed to extract the remainder. One incubation with $N-HClO_4$ at 37° overnight removed all but traces. The acid extracts at room temperature and 37° had ultraviolet absorption spectra similar to that of a yeast ribonucleic acid solution of the same P content in $N-HClO_4$. DNA could not be detected unequivocally by any of the three methods used. The carbohydrate content of the acid extracts was lower than in the corresponding ones from fibre as they contained only starch and no pectin. Nucleotide preparations made by precipitation of the Ba-ethanol insoluble P also contained much less polysaccharide than similar preparations from fibre. The RNA content of the chloroplast fraction from which much of the starch had been removed by differential centrifugation was 30–40 mg./g. of the dry matter.

In the chloroplast fraction which had been incubated in water at 37° the amount of P in the various fractions was greatly diminished owing to the action of ribonuclease and the phospholipid-splitting enzyme. The ribonucleic acid which is not extracted by $N-HClO_4$ at room temperature and which appears to be more firmly bound than the rest, is, however, susceptible to the action of ribonuclease.

Table 7. *Phosphorus fractionation in some sap precipitates*

(200 ml. sap centrifuged at 8000 rev./min. (6000 g) to sediment the chloroplast fraction and the successive precipitates formed after standing for 1 and 3 days at 4°. The chloroplast fraction, with much of the starch removed, and the precipitates were washed and then suspended in water. The P fractions were obtained by extracting successively with 0.2 $N-HClO_4$, ethanol-ether and $N-HClO_4$ at 37°.)

	Chloroplast fraction	Ppt. after 1 day	Ppt. after 3 days
Dry matter (g.)	1.62	0.52	0.48
P (mg./g. dry matter)	2.1	2.7	2.8
N (mg./g. dry matter)	46.7	94.3	135
P % of total P in fraction:			
Soluble in 0.2 $N-HClO_4$	16.1	10.4	6.5
Lipid P	47.0	23.8	3.0
Nucleic acid P	36.9	65.8	90.5

Sap fractions

The P was also fractionated by the $HClO_4$ procedure in the following materials prepared from sap: (1) the precipitate obtained by adding $HClO_4$, to a concn. of 0.2 N , to sap after removal of the chloroplast fraction; (2) the precipitates which separated out from sap (after removal of the chloroplast fraction) on standing at 4°; (3) material which sedimented at 8000 rev./min. from the resuspended sediment from sap which had previously been spun at 40 000 rev./min. No deoxyribose was detected in any of these fractions. The $HClO_4$ precipitate of sap had a low lipid-P content, 5–10% of the total P. One extraction with $N-HClO_4$ at room tempera-

ture removed the greater part of the remaining P, and it was not necessary to incubate at 37° to free the residue of P, a second extraction at room temperature was effective in doing this. Table 7 compares the results of the P fractionation in the chloroplast fraction and the precipitates which appeared in the sap after 1 and 3 days' standing. The lipid P as a percentage of the total P of the fraction decreased in the successive precipitates while the nucleic acid P increased. The material not resuspending after ultracentrifugation had a high P content (over 10 mg./g. of the dry matter), about half of which was nucleic acid P and 40% lipid P. No attempt has been made to fractionate the P in the sap which is not precipitable with acid.

Comparison of methods for estimation of nucleic acids

The method of fractionating RNAP and DNAP used by Schmidt & Thannhauser (1945) on animal tissues and by von Euler & Hahn (1947, 1948) on leaf tissue was tried on several batches of tobacco-leaf fibre and the results compared with the modified Ogur & Rosen method. Incubation of fibre with $N-NaOH$ at 37°, after removal of acid and ethanol-ether soluble P, brought out all the P of the fibre in organic form. However, when fibre was incubated with alkali, even after thorough treatment with solvents to remove pigments, the extract was yellow or brown and unsuitable for colorimetric estimations of inorganic P or of DNA. An added disadvantage was the high N and carbohydrate content of the extracts. The amount of RNAP as a percentage of the total P was higher in the Schmidt & Thannhauser method than in the $HClO_4$ method, while the DNAP was correspondingly lower. This is to be expected because of the DNAP fraction in the $HClO_4$ method containing some RNAP.

FACTORS AFFECTING THE ENZYMIC BREAKDOWN OF PHOSPHORUS COMPOUNDS IN FIBRE, CHLOROPLAST AND SOME SAP FRACTIONS

The release of phosphorus on incubation in water and salt solutions has been observed with fractions from the leaves of a number of species, including tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), bean (*Phaseolus vulgaris* L.), bryony (*Bryonia dioica* Jacq.), comfrey (*Symphytum officinale* L.), groundnut (*Arachis hypogaea* L.), and cabbage (*Brassica oleracea* L.). Most experiments were done with tobacco leaves.

Conditions of incubation

Effect of temperature. At 4° there was a gradual increase in the amount of phosphorus extractable with water. At room temperature the rate of release was increased, and at 37° the action was rapid and substantially complete in a few hours. The greater part of the phosphorus in the extracts was inorganic phosphorus. Fig. 2 shows the effect of temperature on the liberation of P from fibre.

Effect of pH. The optimum pH for the liberation of phosphorus is between 5 and 6. Fig. 3 shows the results of an experiment in which portions of a chloroplast suspension were incubated for 4 hr. at

pH values between 4 and 8. At pH values on the acid side of the optimum the inorganic and total phosphorus in the extracts was of the same order and the amount liberated fell off steeply as the pH became lower. On the alkaline side of the optimum there was a less steep fall in the amount released and the inorganic phosphorus was only half of the total phosphorus in the extracts.

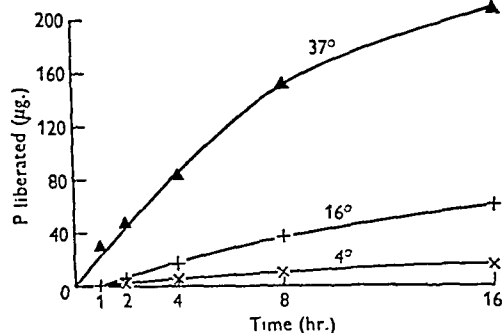


Fig. 2. Effect of temperature on enzymic release of P from fibre. 0.5 g. lots of fibre (dry matter, 100 mg.; P 300 µg.) in 8 ml. 0.025N-NaCl incubated at 4, 16 and 37° for times shown.

Effect of salts. Sodium chloride, sodium citrate and sodium azide increase the rate of liberation of phosphorus. Table 8 shows the effect of various concentrations of sodium chloride and sodium citrate on the release of phosphorus from fibre. Total and inorganic phosphorus liberation were activated by both salts, but a citrate concentration of only about one-twenty-fifth that of the sodium chloride was needed to achieve the same result. Fig. 4 shows the results for three concentrations of sodium azide, compared with sodium chloride, on liberation of total phosphorus from the chloroplast fraction.

The effects due to the activity of ribonuclease and the phospholipin-splitting enzyme can be separated from each other as the phosphorus is split from the RNA much more rapidly than from the phospho-

lipin, particularly in the presence of citrate. The results of an experiment using fibre are given in Table 9. When the phosphorus fractionation in fresh fibre and in fibre incubated for 1 hr. at 37° in

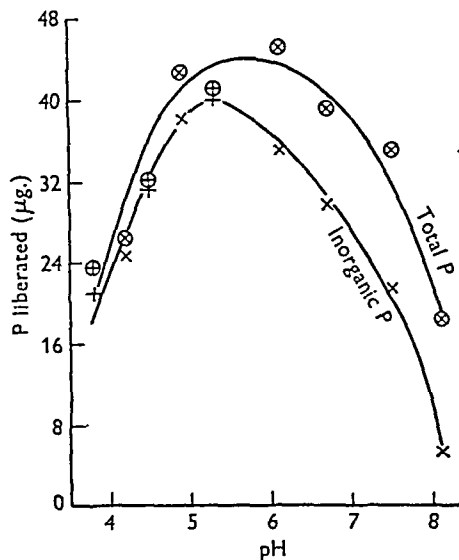


Fig. 3. Effect of pH on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 49 mg.; P, 100 µg.) + 1 ml. buffer solution. The final concentration of the acetate buffers was 0.05M, and the Michaelis sodium diethylbarbiturate-acetate buffers were a 1 in 10 dilution of the stock solution. Incubated for 4 hr. at 37° and 2 ml. 10% (w/v) TCA added before being centrifuged. Inorganic and total P determined in the supernatants. The P values given by a portion of suspension which was not incubated and to which TCA was added were subtracted from the results. ⊗ and ×, Michaelis buffers; ⊕ and +, acetate buffers.

0.05M-sodium citrate buffer (pH 6) is compared (A and B in the table) it is clear that most of the phosphorus in the incubation extract is from the fraction soluble in N-perchloric acid at room

Table 8. *The effect of various concentrations of sodium chloride and sodium citrate on the liberation of phosphorus from fibre*

(0.5 g. lots of fibre (dry matter, 146 mg.; P, 220 µg.) incubated with 5 ml. water or NaCl solution or sodium citrate solution (pH 6) of concn. shown for 2, 4 and 18 hr. at 25°.)

Time of incubation ...	P in extract (µg.)					
	2 hr.		4 hr.		18 hr.	
	Inorganic	Total	Inorganic	Total	Inorganic	Total
Water	15	22	30	30	54	55
NaCl: 0.25 M	42	58	65	63	109	117
0.05 M	25	27	45	49	76	88
0.01 M	20	22	28	38	60	75
Sodium citrate: 0.05 M	80	120	108	127	151	160
0.01 M	39	53	65	72	135	141
0.002 M	24	24	40	49	67	69

Table 9. Separation of the effects due to ribonuclease and the phospholipin-splitting enzyme during incubation of fibre

(3 g. lots of fibre (dry matter 30.2%, P 2.5 mg./g. of dry matter) (A) P fractionated without previous incubation. (B) P fractionated after 1 hr. incubation in 0.05 M-sodium citrate buffer solution (pH 6). (C) Incubated for 1 hr. as (B), extract removed and fibre reincubated for 18 hr. in fresh solution. P fractionated in fibre residue.)

	(A)		(B)		(C)	
	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)
Incubation for 1 hr. at 37°	—	—	1.240	49.5	1.195	48.0
Incubation for 18 hr. at 37°	—	—	—	—	0.508	20.4
0.2 N-HClO ₄ , 25 ml.	0.262	10.5	0.083	3.3	0.083	3.3
Ethanol-ether, 35 ml., i.e. lipid P	0.655	26.2	0.591	23.7	0.161	6.5
N-HClO ₄ , 16°, 25 ml., i.e. RNAP	0.985	39.4	0.117	4.7	0.055	2.2
N-HClO ₄ , 37°, 25 ml., i.e. mainly DNAP	0.497	19.9	0.423	16.9	0.387	15.5

temperature, i.e. RNAP. The lipid phosphorus and DNAP fractions have not been much diminished. When B and C in the table are compared it is seen

phosphorus in the incubation extract is now from the lipid fraction.

Pretreatments

Milling. Grinding the fibre in a triple roller mill (Bawden & Pirie, 1944) did not increase the rate of liberation of phosphorus or the total amount of phosphorus released when the fibre was subsequently incubated in salt solution at 37°.

Boiling. Fibre which had been boiled did not liberate inorganic phosphorus when it was incubated at pH 6, but some phosphorus in organic form was released. Lipid phosphorus did not decrease in amount when boiled fibre was incubated. The addition of dialysed sap or a dialysed extract from fresh fibre caused inorganic phosphorus to be liberated and the total amount of phosphorus brought out was increased. Table 10 shows the effect of adding a dialysed extract to boiled fibre.

Table 10. Effect of adding a dialysed fibre extract to boiled fibre

(0.5 g. lots of fibre (dry matter, 29.3%; P, 2 mg./g. dry matter) + 4 ml. sodium citrate buffer, 0.02 M (pH 6) + 1 ml. water or 1 ml. dialysed extract of fresh fibre. Incubated at 37° for times given. Total and inorganic P determined in the extracts.)

Time (hr.)	P in extract (mg./g. dry fibre)			
	Boiled fibre		Boiled fibre + dialysed extract of fresh fibre	
	Inorganic	Total	Inorganic	Total
1	<0.02	0.146	0.068	0.247
3	—	0.218	0.102	0.314
6	—	0.287	0.222	0.564
22	<0.02	0.486	0.324	0.966

Acid extraction and ethanol-ether extraction. Phosphorus in organic form, but no inorganic phosphorus, was liberated from fibre which had been extracted with dilute acid or with ethanol-ether when it was subsequently incubated at pH 6.

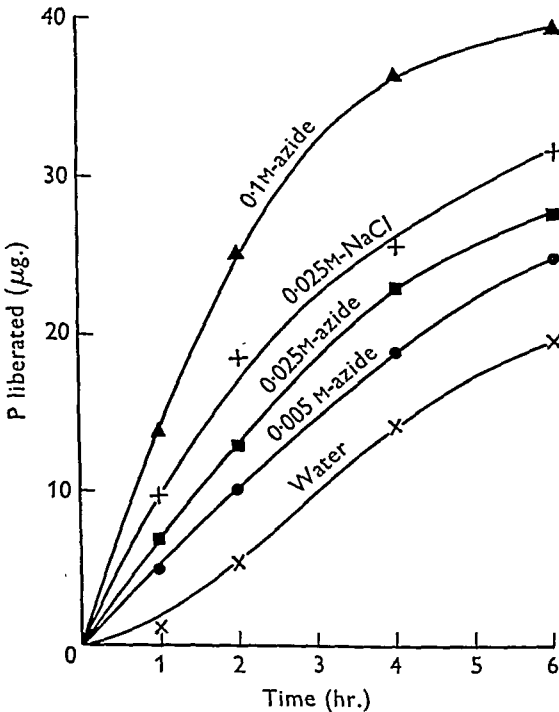


Fig. 4. Effect of sodium azide on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 47 mg.; P, 90 µg.) + 1 ml. water, NaCl or sodium azide solution (with pH previously adjusted to 6.0). Incubated at 37° for times shown and 2 ml. 10% (w/v) TCA added before being centrifuged. Total P determined in the supernatants. The P value given by a portion of suspension which was not incubated and to which TCA was added was subtracted from the results.

that a second incubation for 18 hr., of another portion of fibre, leads to a further diminution in the RNAP fraction, but that the greater part of the

Enzyme activities of fibre extracts

Extracts made by soaking fibre in sodium chloride or sodium citrate buffer (pH 6), followed by dialysis, had ribonuclease and phosphatase activity. Inorganic phosphorus was also split from nucleotide preparations made from fibre and chloroplast fractions. Citrate did not activate the liberation of organic phosphorus from yeast ribonucleic acid by leaf ribonuclease (Pirie, 1951), nor the enzymic liberation of inorganic phosphorus from sodium β -glycerophosphate and nucleotide preparations. Phosphatase and nucleotidase activity was lost by bringing the enzyme solution to the boil, but the ribonuclease is more heat stable and only about half the activity was destroyed by this treatment. Deoxyribonuclease activity was detectable, but with 24 hr. incubation at 37° only 5% of the phosphorus from thymus deoxyribonucleic acid was released compared with 75% from yeast ribonucleic acid. The action of a fibre extract on either lecithin or a phospholipid preparation from leaves has not been tested. The properties of the phospholipid-splitting enzyme are being investigated further.

Composition of incubation extracts

Table 11 gives the composition of a typical incubation extract of fibre. Part of the nitrogen and carbohydrate is present as nucleosides and the remainder as breakdown products of starch and

Table 11. *Composition of an incubation extract of fibre*

(6 g. fibre (1.80 g. dry matter, 58 mg. N, 5.4 mg. P) incubated at 37° in 30 ml. water for 18 hr.)

	Total in extract (mg.)
Dry matter	234
Total P	3.33
Inorganic P	2.64
Nitrogen	9.9
Total carbohydrate	102
Reducing sugar	72

protein due to the activity of the leaf enzymes. No deoxypentose could be determined with certainty in the extracts. The extracts absorbed strongly in the ultraviolet with a maximum at 260 m μ . Adenine, guanine, cytosine and uracil were detected by paper-partition chromatography in the extracts after hydrolysis.

DISCUSSION

The observation that most of the phospholipid of leaf fibre and chloroplast fractions is not extracted with ethanol-ether until after treatment with acid is in agreement with results obtained with animal and some other plant tissues (Lovern, 1942). In animal

tissues there is evidence that lipids are bound because of the formation of lipoprotein complexes but in plants combination with carbohydrates has also been suggested (Jamieson, 1938). Frey-Wyssling (1949) was of the opinion that lipoproteins were present in intact chloroplasts, but on isolation and preparation the lipids dissociated from the protein. It is clear, however, that it is a stable linkage between the phospholipid and protein or other substance to which it is attached.

Hanahan & Chaikoff (1948) found that an enzyme preparation from cabbage leaves acting on soy bean lecithin caused a loss of only 5% of the ether-soluble phosphorus, when 35% of the nitrogen was removed. They suggested that the activity of this enzyme might explain why Chibnall & Channon (1927) isolated a phosphatidic acid from fresh cabbage leaves. In tobacco leaves, and in others including cabbage, phospholipin-splitting enzymes capable of attacking other linkages are also present. The loss of ethanol-ether soluble P from fibre and chloroplast fractions was much greater than the loss of nitrogen on incubation. The different results obtained by Hanahan & Chaikoff might be due to the different substrate used or to a difference in the stability of the enzymes concerned with phospholipin breakdown.

Sodium chloride, sodium citrate and sodium azide have an activating effect on the liberation of phosphorus from fibre and chloroplast fractions similar to that found with the fission of nucleoprotein (Pirie, 1950). Sodium azide appears to act like any other salt. As ribonuclease activity is not much affected by citrate, the effect of citrate on phosphorus liberation may be due to increased accessibility of the nucleic acid. The apparent activation by citrate of nucleotidase action in fibre, which is not observed with nucleotide preparations, is probably due to more substrate being available.

When fibre which had been boiled, or extracted with dilute acid or with ethanol-ether, was subsequently incubated at pH 6 some phosphorus in organic form but no inorganic phosphorus was brought into solution. The release of organic phosphorus was probably due to ribonuclease activity still present in the fibre. The ribonuclease is relatively heat stable but the phosphatase is readily inactivated. Parker (1951) found that TCA precipitates of sap had about 4% of the ribonuclease activity of the original sap, whereas the phosphatase was almost completely destroyed.

The deoxyribonuclease activity of dialysed sap and fibre extracts was so low that it is not unexpected that the DNAP was affected much less than the RNAP during incubation of fibre. The decrease in the DNAP fraction is probably due mainly to the RNAP present in the fraction being removed by ribonuclease action.

During expansion of the leaf there is a large increase in the total amount of phospholipin present. The dry matter has increased in amount so that phospholipid expressed on a dry matter basis remains the same or increases. The total amount of phosphorus in the nucleic acid fractions from fibre remains about the same, so that it shows a decrease per g. of the dry matter. If the weight of DNA per cell is constant in amount as has been found for various animal tissues (Vendrely & Vendrely, 1948) and bacteria (Mitchell & Moyle, 1951), it would not be expected that DNA would vary during expansion of the leaf as cell division is not taking place.

The perchloric acid method of fractionating phosphorus compounds, although not giving a perfect separation of ribo- and deoxyribo-nucleic acids in leaf tissue, is useful in giving an RNA fraction substantially free from DNA, though the DNA fraction contains RNA. The fact that some of the RNA is not extracted by perchloric acid at room temperature, but requires a higher temperature to bring it out, suggests that it might be bound in a different way from the more easily extracted fraction.

Of the total P in the leaf about 30 % is in the form of ribonucleic acid phosphorus, two-thirds of which is in the fibre. Deoxyribonucleic acid phosphorus accounts for about 7 % and lipid phosphorus a further 15 % of the total phosphorus of the leaf.

SUMMARY

1. The phosphorus of tobacco-leaf fibre and chloroplast fractions has been fractionated into acid-soluble phosphorus, lipid phosphorus, ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus. The distribution in leaves of different ages has been compared.

2. Incubation of fibre and chloroplast fractions in water and salt solutions leads to the liberation of up to 80 % of the total phosphorus present, owing to the activity of ribonuclease and a phospholipin-splitting enzyme.

3. The optimum pH for the enzymic liberation of phosphorus is between 5.5 and 6.

4. Sodium chloride, sodium azide and sodium citrate activate the enzymic release of phosphorus.

5. Milling the fibre does not increase the rate of liberation or the total amount of phosphorus released by enzymic action.

6. Boiled fibre, acid-extracted fibre and ethanol-ether extracted fibre on incubation at pH 6 release phosphorus in organic form but no inorganic phosphorus. When incubated in the presence of dialysed sap or a fibre extract, which have ribonuclease and phosphatase activity, inorganic phosphorus is released and the total phosphorus liberated is increased.

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Reactions of Haematin with Peroxides

By JOAN KEILIN*

Biochemical Department, University of Cambridge

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It is well known that hydrogen peroxide reacts with haemoproteins such as methaemoglobin, catalase and peroxidase, giving a series of compounds which can be distinguished by their absorption spectra. In recent years much work has been done on the mechanism of these reactions, and it was found that catalase and peroxidase are each capable of forming at least three distinct complexes with hydrogen peroxide. The properties of these complexes and the literature on the subject of their formation has recently been reviewed by Theorell (1947), Lemberg & Legge (1949), and Keilin & Hartree (1951). Since in all these complexes it is the haematin prosthetic group which reacts directly with the peroxide, it was of interest to find whether free haematin can combine with peroxide. So far the only attempt to study this problem was made by Haurowitz (1937*a, b*), whose observations on the reaction between protohaematin and hydrogen peroxide in pyridine will be discussed later in the light of the findings reported here.

In this paper it is proposed to examine the conditions under which free haematin reacts with hydrogen peroxide and ethyl hydroperoxide and the nature of the products of these reactions, the experiments being carried out both with urohaemin and protohaemin. Urohaemin was selected for this purpose because, being much more soluble than protohaemin on account of its eight carboxyl side chains, it had been found to react with many substances such as sodium hydroxide and amino-acids with much greater ease than protohaemin (Keilin, J., 1949, 1950).

EXPERIMENTAL

Protohaemin. This was prepared from ox or horse blood by the method of Schälfejew (1885).

Urohaematin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The uroporphyrin was esterified, and urohaemin was prepared from the octamethyl ester according to the method of Fischer & Orth (1934). A 6.1×10^{-4} M solution of urohaematin in 0.02 N-NaOH was obtained by dissolving the urohaemin in 5 ml. 0.1 N-NaOH, and the volume was made up to 25 ml. with distilled water.

Hydrogen peroxide. The molarity of H_2O_2 in a solution containing 1 ml. perhydrol in 250 ml. distilled water was determined by titration with standard $KMnO_4$ in the usual way. Further dilutions of the H_2O_2 solution were made as

required daily. A fresh stock solution was prepared and standardized every few days.

Ethyl hydroperoxide. This was prepared and standardized according to the method of Baeyer & Villiger (1901).

Spectroscopic methods. The direct observations of the reactions were carried out with a microspectroscope. The absorption spectra in the visible and violet regions were determined with either the Beckman photoelectric spectrophotometer or the Hilger Uvispek photometer; the latter instrument was used for measuring the high absorption densities in the region of the Soret band. The molecular absorption coefficient ϵ is defined as follows: $\epsilon = E/cl$, where c = molarity of the haematin solution, l = optical depth in cm., and E (extinction) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light respectively.

RESULTS

The absorption spectrum of the urohaematin- H_2O_2 complex

When 0.5 ml. of a 6.1×10^{-4} M solution of urohaematin in 0.2 N-NaOH is treated with an equal volume of 0.27 M- H_2O_2 in the presence of 2 ml. N-NaOH, the colour of the solution at once changes from reddish brown to salmon-pink. At the same time, on direct spectroscopic examination, it is seen that the absorption band of urohaematin at 594 m μ . is replaced by two well defined bands in the green region of the spectrum, of which the β -band is a little stronger than the α -band. This absorption spectrum is, however, of very short duration, and within 45–60 sec. the urohaematin band at 594 m μ . begins to reappear as a shading in the yellow region of the spectrum. This band gradually becomes stronger, and the two bands in the green region diminish in intensity as the complex reverts to free urohaematin. These changes, although they begin to take place very soon after the complex is formed, slow down after about 1.5–2 min. and thereafter progress more slowly so that the peroxide complex takes several hours to revert completely to free urohaematin (Fig. 1). At the same time some molecular oxygen is liberated from the H_2O_2 , and there is also some peroxidatic destruction of the urohaematin itself.

In view of the rapid onset of changes in the absorption spectrum of the complex, a modified technique had to be adopted for recording the spectrophotometric curve and the urohaematin-peroxide complex was freshly made for each point of the curve. The following procedure was found to

* Beit Memorial Research Fellow.

be the most convenient: 0.5 ml. of a $6.1 \times 10^{-4} M$ solution of urohaematin was placed in a 1 cm. cell and diluted with 2 ml. $N-NaOH$ (final concentration of $NaOH = 0.7 N$). After adjusting the instrument at the selected wavelength with the control cell in position, 0.5 ml. of a 0.27M solution of H_2O_2 (450 mol. H_2O_2 /mol. urohaematin) was added at zero time by means of an all-glass syringe fitted with a stainless steel needle and the contents of the cell were stirred with a glass rod. Three to four readings of the extinction were taken within the first 2 min.; readings were then taken at 1 min. intervals for the next 5 or 10 min., so long as O_2 bubbles in the solu-

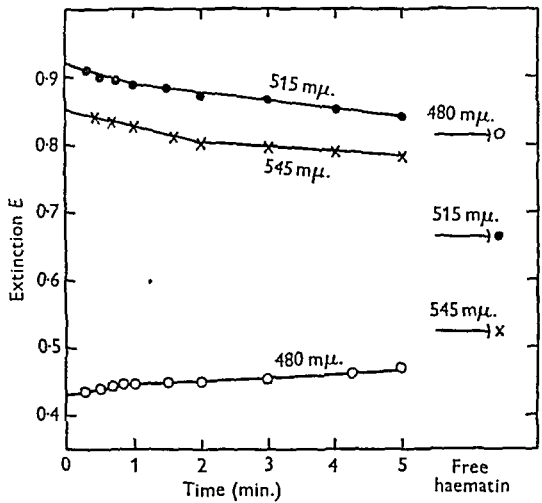


Fig. 1. Reaction of urohaematin with H_2O_2 in 0.7N-NaOH. Typical changes in extinction with time at three wavelengths (515, 545 and 480 mμ.) showing the gradual reversion of the absorption spectrum of the compound to that of free haematin, indicated by the separate points on the right of the figure. The values of $\epsilon \times 10^{-4}$ used for Fig. 2 were obtained by extrapolating back to zero time. (ϵ as defined in text.)

tion did not obscure the optical surfaces of the cell or could be dislodged from them by tapping the cell between readings. The changes of extinction were plotted against time and the spectrophotometric curves were constructed from the extinction obtained by extrapolating back to zero time, assuming the reaction to take place instantaneously on the addition of the hydrogen peroxide to the urohaematin. To determine the absorption spectrum of the Soret band, 0.25 ml. H_2O_2 (750 mol. H_2O_2 /mol. urohaematin) was added to 0.15 ml. urohaematin diluted with 2.6 ml. of the appropriate alkali. The rest of the operation was carried out as described for the visible region of the spectrum.

The spectrophotometric curves of the urohaematin-peroxide compound obtained in this way show that the positions of the two bands in the visible region of the spectrum are α —547.5 mμ. and β —521 mμ., while the values of $\epsilon \times 10^{-4}$ are 0.87 and

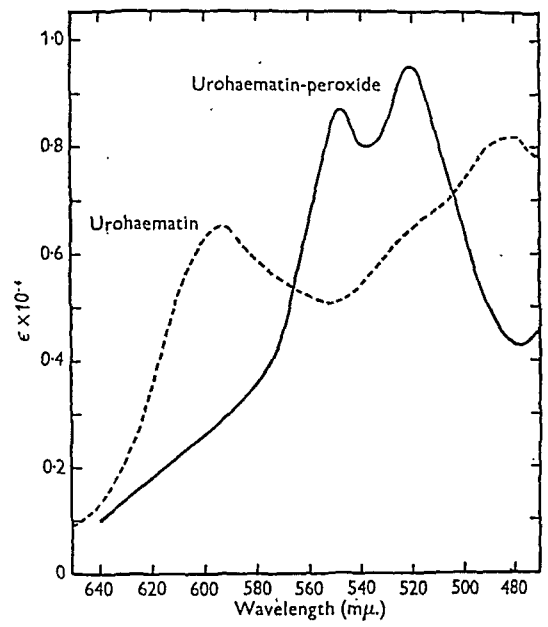


Fig. 2. Absorption bands in the visible region of the spectrum of urohaematin and urohaematin-peroxide in 0.7N-NaOH. (Urohaematin = $1.02 \times 10^{-4} M$; $H_2O_2 = 4.5 \times 10^{-2} M$; $l = 1$ cm.)

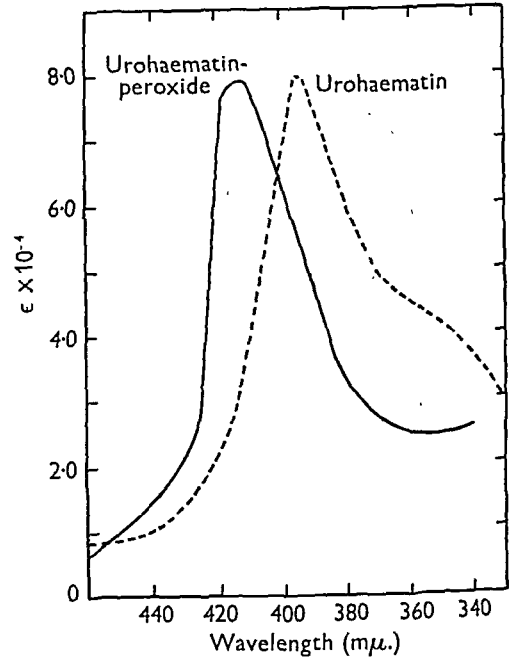


Fig. 3. Absorption spectra showing the Soret bands of urohaematin and urohaematin-peroxide in 0.7N-NaOH. (Urohaematin = $3.05 \times 10^{-5} M$; $H_2O_2 = 2.25 \times 10^{-2} M$; $l = 1$ cm.)

0.95 respectively. In the violet region of the spectrum the Soret band of the urohaematin at 395 mμ. is replaced by the single symmetrical band of the peroxide compound at 413 mμ. ($\epsilon \times 10^{-4} = 7.9$) (Figs. 2 and 3).

Stoichiometric relationships

In order to determine the number of molecules of H_2O_2 combined with each molecule of urohaematin, the urohaematin was treated with various concentrations of H_2O_2 in the presence of 0.7N-NaOH under standard conditions and the percentage formation of the urohaematin-peroxide compound formed in each case was determined as follows. To 0.5 ml. of a $6.1 \times 10^{-4}\text{M}$ solution of urohaematin in 0.02M-NaOH in a 1 cm. optical cell, were added

By plotting $\log [x/(100-x)]$ against $\log [\text{H}_2\text{O}_2]$ a straight line can be obtained which has a slope of n . When $x = 50$,

$$\log [x/(100-x)] = 0 \quad \text{and} \quad \log K = n \log [\text{H}_2\text{O}_2].$$

Thus the value of $\log \text{H}_2\text{O}_2$ when

$$\log [x/(100-x)] = 0, \text{ equals } 1/n \log K.$$

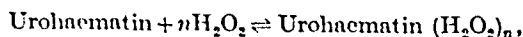
Since n can be calculated from the slope of the line, $\log K$ can be obtained. The position of the line and the values of n and K were obtained by applying the

Table 1. *The relation between the concentration of H_2O_2 and percentage formation of urohaematin-peroxide as determined experimentally*

Molecular ratio H_2O_2 to urohaematin	Formation of complex (%) (x)	Free urohaematin (%) ($100-x$)	$\log \left(\frac{x}{100-x} \right)$	Total H_2O_2 concn. (M)	Free H_2O_2 concn.	
					(M)	($\log \text{M}$)
2	22.8	77.2	-0.5287	2.046×10^{-4}	1.811×10^{-4}	-3.7423
5	38.0	62.0	-0.2125	5.115×10^{-4}	4.707×10^{-4}	-3.3273
10	55.8	44.2	+0.1011	1.023×10^{-3}	9.66×10^{-4}	-3.0150
15	77.1	22.9	+0.5250	1.534×10^{-3}	1.455×10^{-3}	-2.8371

2 ml. M-NaOH and the calculated amount of distilled water required to bring the total volume of the solution to 3 ml. after the addition of the H_2O_2 . A dilute solution of H_2O_2 was introduced into the cell at zero time by means of a syringe so as to provide concentrations of H_2O_2 in the solution ranging from 2.05×10^{-4} to $4.65 \times 10^{-2}\text{M}$, i.e. from 2 to 465 mol. $\text{H}_2\text{O}_2/\text{mol.}$ urohaematin. The extinction at $594\text{ m}\mu$. was then measured at intervals during the first 2 min. and the value at zero time was obtained by extrapolation as described in the previous section. From these values the percentage formation (x) of the peroxide compound at each concentration of H_2O_2 was calculated by means of the formula $x = 100(a-c)/(a-b)$, where a = extinction of free urohaematin, b = extinction of the fully formed urohaematin-peroxide (465 mol. $\text{H}_2\text{O}_2/\text{mol.}$ urohaematin), and c = extinction of the solution under investigation, all the extinctions being measured at the same wavelength ($594\text{ m}\mu$).

If the reaction is represented by the following equation:



then by the law of mass action

$$K = \frac{[\text{Urohaematin}][\text{H}_2\text{O}_2]^n}{[\text{Urohaematin}(\text{H}_2\text{O}_2)_n]}.$$

Hence if x represents the percentage of the total urohaematin which is combined with H_2O_2 , then

$$\frac{1}{K} = \frac{x}{(100-x)[\text{H}_2\text{O}_2]^n},$$

whence

$$\log [x/(100-x)] = n \log [\text{H}_2\text{O}_2] - \log K.$$

method of least squares to the experimental results after a correction had been made for H_2O_2 combined with urohaematin (Table 1, Fig. 4). It was found

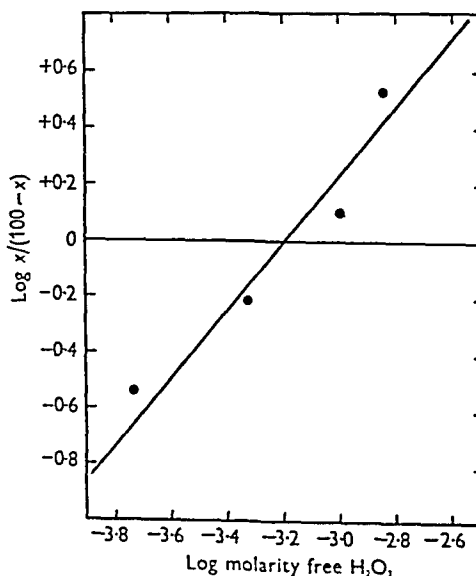


Fig. 4. Relationship between \log molarity H_2O_2 and $\log [x/(100-x)]$, where x = percentage formation of urohaematin-peroxide, and $(100-x)$ = percentage of free urohaematin. The experimental results are indicated by dots. The position of the line was obtained by applying the method of least squares to the experimental results. Correction has been made for the H_2O_2 combined with the urohaematin (Table 1).

that 1 molecule of peroxide is combined per molecule of urohaematin ($n = 1.085$) and the dissociation constant $K = 3.34 \times 10^{-4}\text{M}$ at 20° .

Conditions required for the formation of the urohaematin- H_2O_2 complex

If urohaematin in 0.2N-NaOH is treated with H_2O_2 in the presence of 0.1N-NaOH instead of N-NaOH as described above, the complex formation never proceeds to completion and a shadow due to the persistence of the free urohaematin band is always seen in the yellow region of the spectrum. A spectrophotometric curve constructed under these conditions (total concentration of NaOH = 0.07N) showed that both bands were nearer the blue end of the spectrum and their relative intensities were reversed (α —544.5 $\text{m}\mu$., $\epsilon \times 10^{-4} = 0.9$; β —516 $\text{m}\mu$., $\epsilon \times 10^{-4} = 0.845$). The urohaematin band persisted as a shoulder about 594 $\text{m}\mu$. and from the extinction readings at this wavelength it was calculated that only 83 % of the urohaematin had reacted with H_2O_2 . On the addition of more H_2O_2 to such a solution in an attempt to produce 100 % formation of the complex, the colour of the solution turned to greenish brown and the absorption bands disappeared, indicating that the urohaematin was destroyed.

Similar results are observed using the micro-spectroscope when the total concentration of NaOH is 0.016N or when 0.1 or 0.28M- Na_2CO_3 is used as diluting fluid instead of NaOH. If, however, under these conditions a few drops of N-NaOH are added the complex formation proceeds to completion.

Urohaematin dissolved in 0.1M-phosphate buffer, pH 7.3, fails to react with H_2O_2 and is, instead, rapidly destroyed. The same results are obtained when the solution is made more alkaline by the addition of disodium phosphate or Na_2CO_3 (0.1, 0.28 or 1.4M), but the addition of NaOH to urohaematin in phosphate buffer allows the peroxide compound to be formed as already described.

When urohaematin is dissolved in 10 % (v/v) ammonia, the crimson solution of ammonia uroparahaematin shows a very broad and diffuse absorption band in the green region of the spectrum extending from 580 to 510 $\text{m}\mu$. with a sharper component centred at 540 $\text{m}\mu$. On the addition of H_2O_2 (50 mol. H_2O_2 /mol. urohaematin) the colour of the solution changes to a scarlet red and the two bands of the peroxide compound appear, only to give way in a few minutes to the absorption band of the paraahaematin. If sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) is added to the urohaematin-peroxide compound in ammonia, the intense absorption bands of the ammonia haemochromogen immediately appear, whereas reduction of urohaematin-peroxide in NaOH gives rise to dihydroxyl-haem (Keilin, J., 1949).

Reactions of pyridine uroparahaematin with H_2O_2

Urohaemin is not readily soluble in pyridine, and the pyridine paraahaematin is only obtained in aqueous solution at approximately neutral or slightly acid pH. The red solution then shows the characteristic two-banded spectrum of a paraahaematin in which the β -band is stronger and broader than the α -band (α —555 $\text{m}\mu$., $\epsilon \times 10^{-4} = 0.69$; β —525 $\text{m}\mu$., $\epsilon \times 10^{-4} = 0.92$). On the addition of H_2O_2 to the paraahaematin at about pH 7 the red

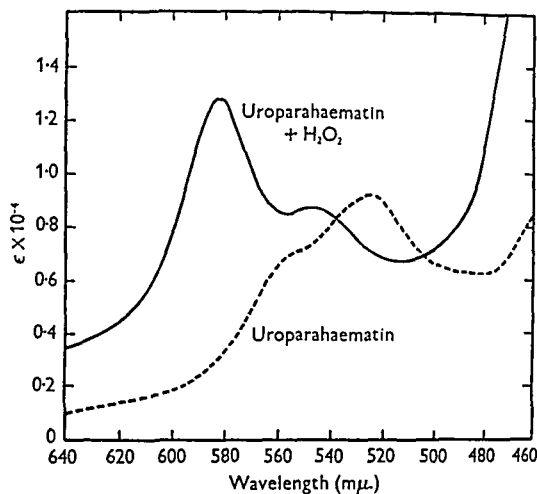


Fig. 5. Absorption bands in the visible region of the spectrum of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2 . (Uroparahaematin = 1.045×10^{-4} M; $\text{H}_2\text{O}_2 = 4.5 \times 10^{-3}$ M; $l = 1$ cm.)

solution quickly turns brown and appears green when viewed in a thin layer. Direct spectroscopic observation shows that this colour change is accompanied by the disappearance of the paraahaematin absorption bands. After a slight lag of about 15 sec. during which no absorption bands are visible, two bands appear and gradually grow in intensity. The α -band at 582 $\text{m}\mu$. becomes very strong while the β -band at 547 $\text{m}\mu$. remains weak (Fig. 5). This absorption spectrum is stable and, so long as excess H_2O_2 is not present to bleach the solution, it may remain for at least 24 hr. At pH 4 these absorption bands appear more intense, while the addition of NaOH causes them to become weaker and finally to disappear. The addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the fully formed green compound at either acid or alkaline pH does not result in the formation of any pyridine haemochromogen, and there is no spectroscopic evidence that the compound combines with CO (either before or after the addition of $\text{Na}_2\text{S}_2\text{O}_4$) or is affected by potassium ferricyanide.

The spectrophotometric curve of the green peroxide-treated paraahaematin ('green compound')

was determined as follows: to 0.5 ml. of 1.045×10^{-4} M-urohaematin in 0.02N-NaOH were added 0.75 ml. of 0.02M-acetic acid, 0.5 ml. pyridine and 0.5 ml. of 0.027M- H_2O_2 and the volume of the solution (containing 50 mol. H_2O_2 /mol. haematin) was brought up to 3 ml. with distilled water; for pyridine uroparahaematin the H_2O_2 was replaced by distilled water. The spectrophotometric curves were determined in the usual way in the visible region, but in the case of the green compounds it was found that,

be fully discussed later, it was decided to re-examine the effect of H_2O_2 on protohaematin in pyridine under various conditions.

Protohaemin dissolves readily in pyridine giving a red parahaematin with the usual two-banded absorption spectrum (α —560 $\text{m}\mu$., β —530 $\text{m}\mu$.). On the addition of H_2O_2 (perhydrol diluted as required with pyridine), the solution becomes greenish brown and a diffuse absorption band appears at about 605 $\text{m}\mu$. while the α -band of the

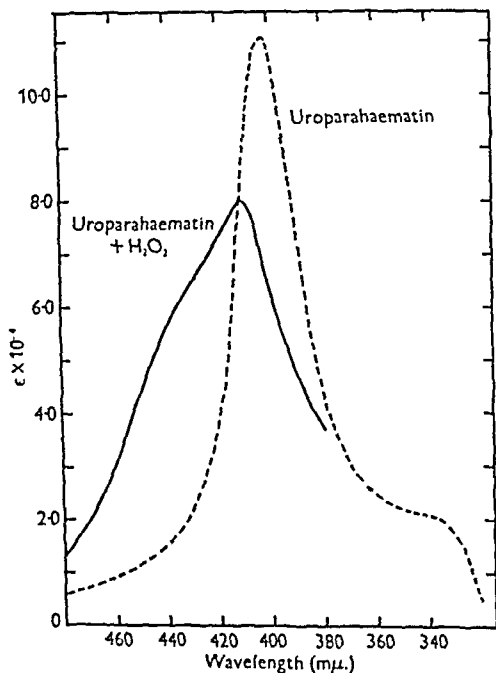


Fig. 6. Absorption spectra showing the Soret bands of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2 . (Uroparahaematin = 1.016×10^{-4} M; H_2O_2 = 2.97×10^{-3} M; $l = 1$ cm.)

at the much greater dilution required for measuring the Soret band, this band was very evanescent. A fresh solution therefore had to be prepared for measuring each point of the curve when determining the height of the Soret band and the peroxide was added last to the cell by means of a syringe as described earlier in this paper. Even with these precautions it was not possible to obtain reliable extinction readings. As seen in Fig. 6, the asymmetric Soret band of the 'green compound' has a main peak at 410 $\text{m}\mu$. ($\epsilon \times 10^{-4} = 8.0$ approx.) and a shoulder at 435 $\text{m}\mu$. which may be due to some intermediate compound.

Reactions of protohaemin and H_2O_2 in pyridine

In view of the differences between the reactions of urohaematin with H_2O_2 described above and the results obtained by Haurowitz (1937 *a, b*) which will

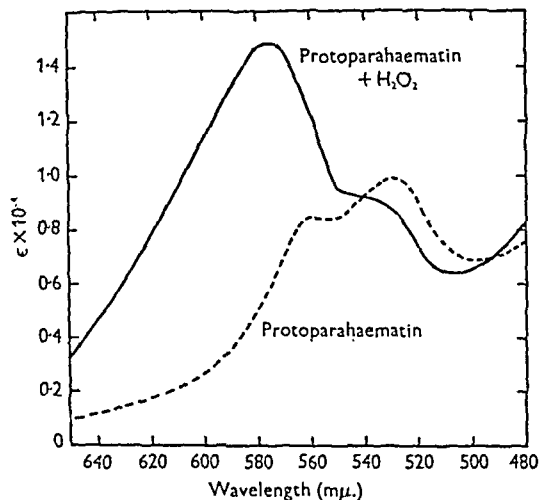


Fig. 7. Absorption bands in the visible region of the spectrum of pyridine protoparahaematin and the green compound obtained on treating it with H_2O_2 . (Protoparahaematin = 1.32×10^{-4} M; H_2O_2 = 1.86×10^{-2} M; $l = 1$ cm.)

parahaematin becomes stronger and the β -band at 530 $\text{m}\mu$. becomes weaker. The band in the red then tends to become stronger and broader and to move towards the blue end of the spectrum so that its centre lies at about 583 $\text{m}\mu$. The general appearance is therefore of a two-banded spectrum with a broad, strong band at 583 $\text{m}\mu$. and a weaker band at 560 $\text{m}\mu$., but the exact positions of these bands depend on both the pH of the solution and the amount of H_2O_2 added. Thus at pH 6.5 the solution is green with red dichroism; the α -band is markedly asymmetric and appears to extend from 570 to 600 $\text{m}\mu$. on direct spectroscopic examination, though the spectrophotometric curve shows that the peak lies at 574 $\text{m}\mu$. (Fig. 7). A weak β -band lies at 535 $\text{m}\mu$., while in the violet region the Soret band of the parahaematin at 403.5 $\text{m}\mu$. is replaced by the band of the 'green compound' at 426 $\text{m}\mu$. On reduction with $\text{Na}_2\text{S}_2\text{O}_4$ a haemochromogen (α —558 $\text{m}\mu$., β —530 $\text{m}\mu$.) is formed by that part of the protohaem which has not been irreversibly altered by the peroxide. With greater concentrations of H_2O_2 the bands at 583 and 560 $\text{m}\mu$. appear more

directly and soon disappear due to the destruction of the protohaematin. Under such conditions, therefore, no haemochromogen is obtained on the addition of $\text{Na}_2\text{S}_2\text{O}_4$.

Reactions between ethyl hydroperoxide and haematin

Ethyl hydroperoxide (EtOOH) reacts with haemoproteins giving, in most cases, complexes which are analogous to those obtained with H_2O_2 , so it was of interest to examine the effect of EtOOH on uro- and proto-haematin.

With urohaematin in NaOH , EtOOH gave an unstable red compound with two absorption bands in the visible region of the spectrum in the same positions as those of the urohaematin- OOH compound. This spectrum could already be observed in solutions containing 0.02N- NaOH , but the concentration of NaOH in the solution had to be raised to 1.5N before the compound was completely formed, as shown by the disappearance of the band of free

strong, wide β -band at 546 $\text{m}\mu$. The reduction of urohaematin- OOEt to dihydroxyl-urohaem can, of course, be brought about at once by the addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the compound.

With pyridine uroparahaematin, EtOOH reacted somewhat differently from H_2O_2 . The scarlet colour of the paraahaematin gave way to a greenish brown; at the same time the absorption bands of the paraahaematin became diffuse, disappeared and were replaced by two bands, a strong α -band at 575 $\text{m}\mu$. and a weak β -band at 537 $\text{m}\mu$. After standing a few minutes (or immediately on the addition of $\text{Na}_2\text{S}_2\text{O}_4$) these bands were replaced by a wide asymmetric α -band lying at 560 $\text{m}\mu$. and a weak β -band at 527 $\text{m}\mu$. belonging to a reduced compound. This absorption spectrum remained unchanged in the presence of CO thus indicating that it was not a true haemochromogen, and it reverted to the oxidized form on the addition of potassium ferricyanide (Table 2).

Table 2. *Positions and extinction coefficients of the α -, β - and γ -bands of the compounds of urohaematin and protohaematin with H_2O_2 and ethyl hydroperoxide*

Compound	α -Band		β -Band		γ -Band	
	Wavelength ($\text{m}\mu$.)	$\epsilon \times 10^{-4}$	Wavelength ($\text{m}\mu$.)	$\epsilon \times 10^{-4}$	Wavelength ($\text{m}\mu$.)	$\epsilon \times 10^{-4}$
Urohaematin in 0.7N- NaOH	594	0.65	483	0.82	395	8.0
Urohaematin in 0.7N- $\text{NaOH} + \text{H}_2\text{O}_2$	547.5	0.87	521	0.95	413	7.9
Urohaematin in 1.5N- $\text{NaOH} + \text{EtOOH}$	547.5*	—	521*	—	—	—
Reduction compound of urohaematin in 1.5N- $\text{NaOH} + \text{EtOOH}$	578*	—	546*	—	—	—
Dihydroxyl urohaem†	578	0.76	546	1.4	435	11
Pyridine uroparahaematin	555	0.69	525	0.92	402	11.05
Pyridine uroparahaematin + H_2O_2	582	1.27	547	0.87	410	8.0
Pyridine uroparahaematin + EtOOH	575*	—	537*	—	—	—
Reduction compound of pyridine uroparahaematin + EtOOH	560*	—	527*	—	—	—
Pyridine urohaemochromogen (pH 7)	550*	—	520*	—	—	—
Pyridine protoparahaematin	(560)	0.84	530	0.99	403.5	6.55
Pyridine protoparahaematin + H_2O_2 (pH 8 approx.)	{ 605)*	—	560	—	—	—
Pyridine protoparahaematin + H_2O_2 (pH 6.5)	{ 583)*	—	535	0.91	426	5.0
Pyridine protoparahaematin + EtOOH (pH 7.8)	{ (605)*	—	530*	—	—	—
Pyridine protohaemochromogen	{ 562*	—	530*	—	—	—
	558*	—				

* Positions of bands determined with microspectroscope.

† Keilin, J., 1949.

urohaematin at 594 $\text{m}\mu$. Increasing the amount of EtOOH at lower concentrations of NaOH merely led to the destruction of the urohaematin.

When the red urohaematin- OOEt compound was formed under optimal conditions and allowed to stand, the following changes took place within about 3 min. The absorption bands of the compound faded, the solution becoming orange-pink in colour, and they were then gradually replaced by the characteristic absorption spectrum of the red ferrous compound, dihydroxyl-urohaem, which consists of a narrow, weak α -band at 578 $\text{m}\mu$. and a

Since ethyl hydroperoxide decomposes spontaneously to give acetaldehyde and water, it was necessary to examine the action of acetaldehyde on various urohaematin compounds before attributing to EtOOH the spontaneous reductions of urohaematin- OOEt and the green compound given by the paraahaematin. Acetaldehyde was therefore added to alkaline urohaematin and to pyridine uroparahaematin but no changes were observed in the absorption spectra of these compounds.

In the case of protohaematin no reaction was observed between EtOOH and alkaline protohaematin,

although various concentrations of NaOH and both aqueous and ethanolic solutions of protohaematin were examined. Ethanolic solutions were investigated, since it appeared likely that a haematin requires for its reaction with peroxide the same conditions as are necessary for it to give a dihydroxyl haem in its reduced state (protohaematin can only react with NaOH to give its dihydroxyl compound in the presence of a solvent such as ethanol). This point will be discussed more fully later.

Ethyl hydroperoxide did, however, react with pyridine protopara-haematin. At pH 7.8 the para-haematin solution became greenish brown on the addition of EtOOH and an absorption band was seen for about 30 sec. at 605 m μ . Meanwhile, the band at 560 m μ . became stronger and the para-haematin spectrum was spontaneously replaced by that of a fairly stable green compound with α - and β -bands at 562 and 530 m μ . respectively, the change being complete within a few minutes. This compound, which failed to react with CO and was not affected by Na₂S₂O₄, is probably analogous to the reduction compound given by pyridine uropara-haematin and EtOOH. If less EtOOH were used, the reaction was complicated by the final appearance of a haemochromogen (α —558 m μ ., β —530 m μ .) given by some of the haematin which had not been altered by EtOOH. This haemochromogen reacted normally with CO.

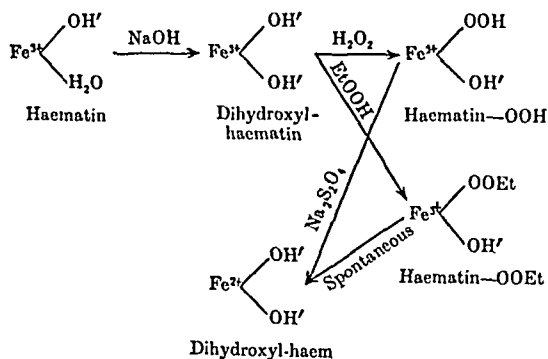
DISCUSSION

Urohaematin, although very freely soluble in all dilute alkalis and alkaline buffer solutions, was found to react with H₂O₂ and with ethyl hydroperoxide to form reversible compounds only when excess hydroxyl ion was present as in sodium hydroxide solutions. A well defined, though unstable, compound was then formed with two absorption bands in the visible region of the spectrum (α —547.5 m μ ., β —521 m μ .). This absorption spectrum was of relatively short duration, and in the case of the H₂O₂ compound it reverted to that of free urohaematin. Titration of urohaematin with H₂O₂ showed that one molecule of H₂O₂ is combined per molecule of urohaematin.

It is now generally accepted that in alkaline haematins the iron atom is co-ordinated in positions 5 and 6 with one hydroxyl group and one molecule of water (Davies, 1940; Shack & Clark, 1947), whereas in haems (ferroporphyrins) these places are occupied by 2 molecules of water. It was recently shown (Keilin, 1949) that under certain conditions in the presence of NaOH, haems react with the hydroxyl ion giving dihydroxyl-haems in which two hydroxyl groups are co-ordinated per atom of haem iron.

Since, for the formation of the peroxide compound, urohaematin also requires the presence of excess

hydroxyl ion, and reduction of the urohaematin-peroxide compound with Na₂S₂O₄ gives dihydroxyl-urohaem, it is suggested that a dihydroxyl-urohaematin may be an intermediate in the reaction between urohaematin and H₂O₂, one molecule of H₂O₂ replacing a hydroxyl group on the urohaematin iron. As ethyl hydroperoxide gives a compound with alkaline urohaematin analogous to that given by H₂O₂ and under the same conditions, one may assume that the mechanism of formation of the peroxide compound is the same in each case. It was found, however, that urohaematin-OOEt is spontaneously reduced to dihydroxyl-urohaem in a few minutes. Since acetaldehyde, the decomposition product of ethyl hydroperoxide, cannot cause this reduction one must conclude that it is probably due to the reducing action of ethyl hydroperoxide itself. As there is no ferrous form of urohaematin-peroxide a change in the valency of the urohaematin iron must result in the formation of dihydroxyl-urohaem. In the following scheme, which summarizes these reactions, the lines joining the urohaematin iron to the reacting groups do not indicate the nature of the valency bonds and the four valencies directed towards the pyrrol nitrogen atoms are omitted.



So far a protohaematin-peroxide compound analogous to that given by urohaematin has not been obtained, but this does not preclude its existence and it is possible that the conditions for its formation may yet be found.

Haurowitz (1937 *a*, *b*), in his important investigations on the reactions between protohaematin and H₂O₂ in pyridine, described a transient two-banded absorption spectrum (α —582 m μ . and β —573 m μ . approx.) which was replaced by a single asymmetrical band in the visible region at 575 m μ . On the basis of these absorption bands Haurowitz postulated the existence of two protohaematin peroxide compounds in which a molecule of H₂O₂ replaced in turn each of the 2 molecules of pyridine of the para-haematin, thus giving a 'primary' and 'secondary' compound respectively.

It was mentioned earlier that in alkaline solution the addition of H_2O_2 to pyridine-protoparahaematin caused an absorption band to appear at about 583 $\text{m}\mu$, while the parahaematin α -band at 560–565 $\text{m}\mu$ was reinforced and, together with the new band, presented the appearance of a two-banded spectrum. It is probably this absorption spectrum which was observed by Haurowitz but ascribed by him to the 'primary compound'. The band at 575 $\text{m}\mu$, which he attributed to the 'secondary compound', corresponds to that given best by proto-parahaematin with H_2O_2 in neutral or slightly acid conditions (Fig. 7); the fact that this band is asymmetrical and is shifted nearer to the blue end of the spectrum than that of the green compound given by uroparahaematin with H_2O_2 may indicate that it is due to a reduction compound similar to that given by ethyl hydroperoxide with both uro- and proto-parahaematin.

The actions of H_2O_2 on the pyridine-parahaematin of uro- and proto-haemins resulting in the formation of the green compounds are completely different from their actions on urohaematin in NaOH . The non-reversibility of formation of the green compounds, together with the fact that when fully formed they fail to give typical pyridine haemochromogens on the addition of $\text{Na}_2\text{S}_2\text{O}_4$, suggests that the haematin has been irreversibly modified in some way, although the presence of a Soret band indicates that the ring has not actually been opened. These green compounds therefore cannot be considered as haematin-peroxide compounds in which the peroxide is co-ordinated with the haematin iron, as was suggested by Haurowitz, but more probably belong to the oxyporphyrin class of pigments.

The reducing action of a peroxide was first described by Kuhn & Wassermann (1933), who showed that ferric salts can be reduced by H_2O_2 to ferrous salts which are detected by their ability to combine with 2:2'-dipyridyl to give the well known red complex. In the course of their work on azide catalase, Keilin & Hartree (1945) showed that H_2O_2 can reduce azide catalase to a well defined ferrous compound, and recently they have shown that the methaemoglobin-peroxide complex may, by further treatment with H_2O_2 , be reduced to haemoglobin which combines with O_2 liberated during the reaction to form oxyhaemoglobin (Keilin & Hartree, 1951). However, on treating methaemoglobin with very small concentrations of H_2O_2 below pH 5, the absorption bands of methaemoglobin disappeared and were replaced by a wide band at 590 $\text{m}\mu$, belonging to a new compound showing red-green dichroism, which could be reduced by $\text{Na}_2\text{S}_2\text{O}_4$ to haemoglobin. From a consideration of its spectroscopic properties, Keilin & Hartree concluded that it was an oxidation product of haemoglobin in which

the haematin ring, though still intact, was modified, probably at one of the methine bridges. This compound bears a strong resemblance to that obtained by treating pyridine protohaematin with H_2O_2 . Thus H_2O_2 can be both a reducing agent and an oxidizing agent in the same system, acting on different parts of the molecule according to the conditions of the experiment.

So far most work has been done on the complex reactions between the haemoproteins catalase, peroxidase and methaemoglobin with H_2O_2 , but free protohaematin, the prosthetic group of these haemoproteins, was not known to react with H_2O_2 in any way except when co-ordinated with pyridine. It has now been shown that the compound formed in this reaction is merely an oxidation product of haematin and is not a true haematin peroxide compound such as it is necessary to visualize in the biological systems mentioned. Urohaematin, however, probably by virtue of being fully dispersed in solution, forms a true compound with H_2O_2 and with ethyl hydroperoxide and so provides a model for a free haematin peroxide compound where the peroxide is co-ordinated with the iron while the porphyrin ring remains unaltered.

SUMMARY

1. Urohaematin is shown to combine with hydrogen peroxide and ethyl hydroperoxide in the presence of excess hydroxyl ion. For complete formation of the complex the total concentration of sodium hydroxide required was 0.7N in the case of hydrogen peroxide and 1.5N for ethyl hydroperoxide. The effect of hydrogen peroxide on urohaematin in other alkalis is discussed.

2. The urohaematin-peroxide complex has a characteristic absorption spectrum (α —547.5 $\text{m}\mu$; β —521 $\text{m}\mu$; γ —413 $\text{m}\mu$) which is of short duration. In the case of hydrogen peroxide the absorption bands of free urohaematin begin to replace it within a few seconds while with ethyl hydroperoxide the compound is reduced to dihydroxyl-haem.

3. In the urohaematin-peroxide compound one molecule of hydrogen peroxide is combined per molecule of urohaematin. The dissociation constant $K = 3.34 \times 10^{-4} \text{ M}$ at 20°.

4. It is suggested that dihydroxyl-urohaematin may be an essential intermediate for the subsequent formation of the urohaematin-peroxide compound, one molecule of hydrogen peroxide or ethyl hydroperoxide replacing one hydroxyl group on the urohaematin iron atom.

5. A protohaematin-peroxide compound has not so far been obtained with either hydrogen peroxide or ethyl hydroperoxide.

6. The pyridine parahaematin of both uro- and proto-haematin react with hydrogen peroxide and

ethyl hydroperoxide to give 'green compounds' with two-banded absorption spectra. The exact positions of these bands depend on the pH and the amount of peroxide added. In the case of protopara-haematin additional transient absorption bands are observed under certain conditions.

7. The 'green compound' given by pyridine uropara-haematin and ethyl hydroperoxide reduces spontaneously and reversibly to a compound which differs from pyridine urohaemochromogen in that the positions of its absorption bands are nearer the red and in that it does not combine with carbon monoxide. The green compounds given by pyridine protopara-haematin with hydrogen peroxide (pH 6.5)

and ethyl hydroperoxide are probably of the same nature.

8. It is suggested that these 'green compounds' correspond to those given by pyridine protopara-haematin and hydrogen peroxide described by Haurowitz, but that they are oxidation products of the haematins and not compounds of the haematins with hydrogen peroxide.

9. Urohaematin-peroxide is so far the only example of a free haematin-peroxide compound where the porphyrin ring is unaltered.

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A New Amino-acid Amide in the Groundnut Plant (*Arachis hypogaea*): Evidence of the Occurrence of γ -Methyleneglutamine and γ -Methyleneglutamic Acid

BY J. DONE AND L. FOWDEN*

Human Nutrition Research Unit, Medical Research Council Laboratories,
 London, N.W. 3

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A brief account of preliminary studies of a new metabolite detected in groundnut plants has already been given (Done & Fowden, 1951): The substance was isolated and found to be an unsaturated amino-acid amide. This earlier work is now described in more detail, together with subsequent investigations of the structure of the new compound.

The first material examined was the 'sap' produced when the turgid stems of young seedlings were incised between the cotyledons and the first leaf. About 20 μ l. of liquid exuded from the cut surfaces. It was found by paper chromatography that the main ninhydrin-reacting component of the exudate gave an orange-brown spot ($R_F = 0.66$ on

chromatograms run in phenol-ammonia). The colour of the ninhydrin spot varied somewhat on different occasions, but always it was clearly outside the range of colours obtained with known α -amino-acids.

A large number of exudates produced in the manner described have been examined, and the new substance (I) has invariably been the dominant ninhydrin-reacting component. When amounts of the order of 20 μ l. were chromatographed, much smaller spots due to asparagine, glutamine, aspartic acid, glutamic acid, alanine and other amino-acids sometimes appeared.

(I) has also been detected in homogenates of stems, leaves, hypocotyls and roots, but in these many other ninhydrin-reacting compounds were also present. The new compound could not be

* Present address: Department of Botany, University College, London, W.C. 1.

detected in seedlings less than 2 days after germination, but it occurred in all samples taken after the differentiation of the first leaf, e.g. in the exudate formed after the incision of the petiole of the fifteenth leaf of a main stem, and in the gynophores bearing immature fruits. The stem, root and hypocotyl of seedlings germinated and maintained in the dark for 12 days all contained (I). It was not detected in immature or ripe seeds, or in the hydrolysate of a commercial sample of arachin. Thus, the compound (I) appeared after germination of the seed and occurred in all the vegetative tissues examined.

A number of varieties of beans, peas and lupins, and the sprouts of potato tubers were examined, but no compound giving the type of ninhydrin reaction shown by (I) was observed on the chromatograms.

Preliminary examination of the chemical properties of (I)

The action of hydrochloric acid. Exudate was heated with 5N-hydrochloric acid at 105° for 16 hr. in a sealed tube; hydrochloric acid was removed by evaporation. (I) could not be detected on chromatograms, but an intense yellow-brown spot ($R_F = 0.30$ in phenol-ammonia) appeared. Here again, the colour of the spot was quite distinct from those obtained with known α -amino-acids. Similar results were obtained after treatments with 5N-hydrochloric acid for 3 days at 105° and with N-hydrochloric acid for 3 hr. at 100°. Spots due to asparagine and glutamine occurred on chromatograms of the exudate, but the intensity of each of these was less than one-tenth of the intensity of the main spot due to (I). These amides were hydrolysed to the corresponding acids by the hydrochloric acid treatments.

When a sample of exudate containing 6.74 μg . N/ μl . was treated with N-hydrochloric acid for 3 hr. at 100°, ammonia nitrogen equivalent to 2.7 μg ./ μl . was produced. The conditions of hydrolysis in this experiment were those used in the estimation of amide nitrogen (Borsock & Dubnoff, 1939), so the results could be explained by the hypothesis that amide I \rightarrow acid II + NH_3 . This was supported by the chromatographic evidence. The ratio of the R_F of (I) to that of (II) in phenol-ammonia was 2.2. The corresponding ratios for asparagine and glutamine are 2.5 and 2.0 respectively (Dent, 1948).

(I) and (II) both combined with copper as shown by the copper acetylacetonate test (Wieland & Fischer, 1948); thus the presence of α -amino nitrogen was indicated.

Autolysis. When homogenized leaf tissue was allowed to autolyse for 3 hr. at 37° chromatograms showed that (I) had been converted to (II). Both compounds occurred in ethanolic extracts of fresh plant 'tops'.

Isolation of compound (I). (I) was retained when diluted exudates were run through a column of Zeo-Karb 215 (Partridge & Westall, 1949) and the compound was isolated after elution with ammonia solution. It was readily soluble in cold water and formed colourless plates on crystallization from aqueous ethanol. Elementary analysis gave an empirical formula $\text{C}_6\text{H}_{10}\text{O}_3\text{N}_2$. Half the nitrogen was released as ammonia by treatment with N-sodium hydroxide for 24 hr. at room temperature, and by treatment with N-hydrochloric acid for 3 hr. at 100°.

Isolation of compound (II). De-Acidite B (Partridge & Brimley, 1949) retained compound (II) when an autolysate of the plant 'tops' was run through a column of the material. The new acid was eluted by acetic acid followed by dilute hydrochloric acid, and it was isolated by recrystallization of the solid left after evaporation of fractions of the effluent from the column. The compound was only slightly soluble in cold water but much more soluble in hot water. It was therefore convenient to crystallize the compound from hot water. Elementary analysis gave an empirical formula $\text{C}_6\text{H}_9\text{O}_4\text{N}$. All the nitrogen was amino nitrogen as measured by the Van Slyke nitrous acid method. The equivalent weight found by titration with sodium hydroxide was 158 (formula weight 159). No optical activity was detected in an aqueous solution of the sodium salt.

The structure of the new compounds

The analyses of the new compounds confirmed the chromatographic evidence that (II) was an α -amino-acid derived from (I) by deamidation.

The presence of a double bond. Both compounds decolorized a solution of potassium permanganate at room temperature. Treatment with ozone gave a substance which could not be distinguished from aspartic acid by paper chromatography. The new amide therefore appears to resemble asparagine and glutamine in that the amide group is not attached to the α -carbon atom.

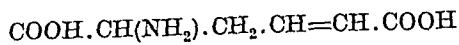
The product of the action of ozone on (II) was isolated and shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein, Nemshinskaya & Vilenkina, 1947).

When (II) was hydrogenated in the presence of palladium-on-carbon catalyst, the amount of gas absorbed corresponded to that required for a reaction $\text{C}_6\text{H}_9\text{O}_4\text{N} \rightarrow \text{C}_6\text{H}_{11}\text{O}_4\text{N}$.

The reduced form of (II), compound (III). Elementary analysis of (III) gave the empirical formula $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$. The compound formed a purple spot with ninhydrin at R_F 0.42 on chromatograms run in phenol-ammonia. The aqueous solution showed no optical rotation.

Since aspartic acid was produced by the action of ozone on (II), it seemed likely that the compound

contained $\text{COOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2\text{C}=\text{}$, and since a titratable carboxyl group has also been detected, possible formulae were



and



The corresponding saturated compounds are α -aminoadipic and γ -methylglutamic acids respectively.

Substance (III) differed from DL- α -aminoadipic acid in its solubility and melting point and in its position on paper chromatograms. α -Aminoadipic acid is converted to ornithine by hydrazoic acid (Adamson, 1939). However, treatment of (III) with hydrazoic acid gave a substance which could be separated from ornithine on chromatograms run in aqueous *n*-butanol-acetic acid mixture (Partridge, 1948). It was therefore evident that (III) was not DL- α -aminoadipic acid.

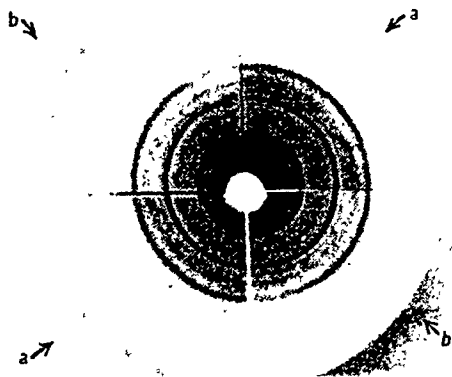


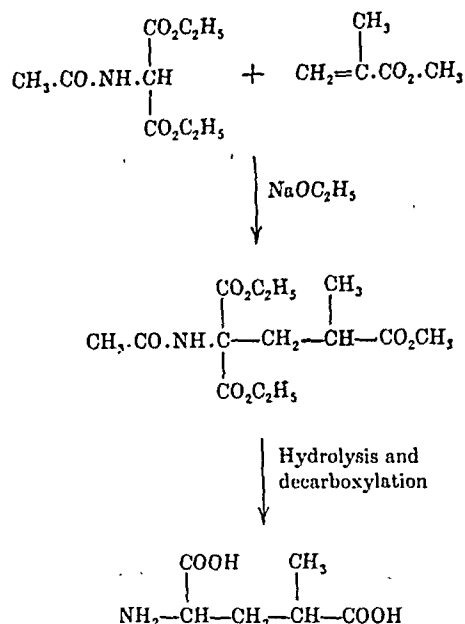
Fig. 1. X-ray powder photographs of (a) compound (III) and (b) synthetic γ -methylglutamic acid. Cu (Ni filtered) $\kappa\kappa$ radiation; specimen to film distance 5 cm.

On the other hand, it had been found that the melting point of (III) was low compared with those of most of the known α -amino-acids, and pyrrole derivatives had been detected in the vapour formed when (III) was fused with zinc dust. These facts suggested that the compound contained an α -amino and a γ -carboxyl group, and it was considered that (III) might be γ -methylglutamic acid.

Synthesis of γ -methylglutamic acid. Glutamic acid has been synthesized by Michael condensations: methyl acrylate and ethyl phthalimidomalonate were employed by Marvel & Stoddard (1938), and methyl acrylate and ethyl acetaminomalonate by Snyder, Shekelton & Lewis (1945).

Evidence of the synthesis of (III) by condensation of methyl methacrylate with ethyl phthalimidomalonate was obtained. However, better results

were obtained by the reaction between methyl methacrylate and ethyl acetaminomalonate:



Elementary analysis of the synthetic material gave the empirical formula $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$; when fused with zinc dust it gave a positive pine-splinter test for pyrrole derivatives.

The position of the synthetic compound on paper chromatograms coincided with that of the natural acid (III). Both substances melted at 154 – 155° and the melting point of a mixture was unchanged.

Prof. Kathleen Lonsdale, F.R.S., very kindly arranged for X-ray powder photographs of the natural and synthetic acids to be prepared (Fig. 1). She reported that the two samples gave identical results.

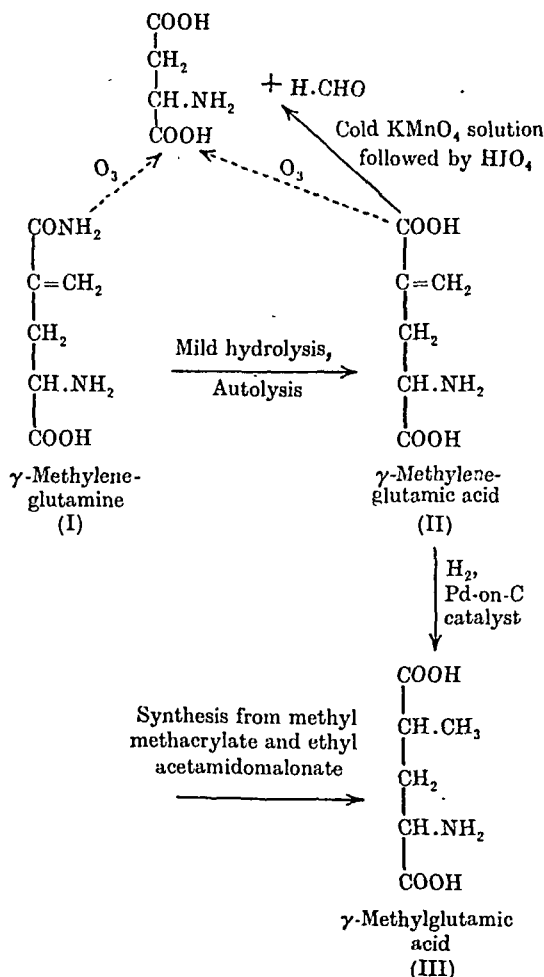
Oxidative degradation of (II). The synthesis of (III) had provided good evidence of the carbon skeleton of the new compounds. The possible position of the double bond had been indicated by the formation of aspartic acid by treatment of the unsaturated compounds with ozone. However, the yield of aspartic acid was low, and no non-nitrogenous fragment had been isolated. Accordingly, further experiments were made on the oxidative degradation of (II).

The compound reacted almost completely with an approximately equimolecular amount of potassium permanganate in ice-cold aqueous solution. Further oxidation with periodic acid gave, on different occasions, yields of formaldehyde (dime-done derivative) of 41, 27 and 25% (mol. basis). The main ninhydrin-reacting component resulting from permanganate-periodate oxidation could not be separated from aspartic acid on chromatograms run in phenol-ammonia, butanol-acetic acid

(Partridge, 1948) or collidine-lutidine solvents. Evidence that aspartic acid was formed in 32% yield was obtained by a quantitative chromatographic method (Fowden, 1951).

Exhaustive methylation (Braunstein *et al.* 1947) of the product of oxidative degradation after removal of formaldehyde gave fumaric acid, corresponding to a yield of 29% of aspartic acid.

Provisional structures of the new compounds are included in the following scheme, which also summarizes the main reactions described:



EXPERIMENTAL

All melting points are uncorrected.

Isolation of compound (I). Young plants (3000–4000) were grown in boxes containing either garden soil or gravel and nutrient salts. Exudates were obtained by cutting the main stems when four to eight leaves had developed. They were collected in capillary tubes, and stored in the refrigerator in 2–4 ml. batches. The precipitate which formed was removed by centrifugation. A total of approx. 40 ml. of material was obtained. Preliminary attempts to concentrate or crystallize (I) by extraction of dried exudate with ethanol and by other methods were not successful.

A column of Zeo-Karb 215 was prepared (Partridge & Westall, 1949), 15 cm. in length and 0.9 cm. in diameter. The exudate material was diluted with 250 ml. of water and passed through the column at the rate of 1 drop in approx. 4 sec. The resin darkened progressively from the top as the mixture passed through the column, until finally only a few mm. at the bottom remained unchanged. Distilled water (1 l.) was used to wash the column and then a solution of 0.15N- NH_3 was run through. Eighteen fractions of vol. 10–15 ml. were collected.

A light-coloured band moved down the column during elution. This was apparently due to (I), since when it 'broke through' fraction 9, 5 μl . of the effluent gave a strong yellow to red colour when treated with ninhydrin on filter paper. Chromatograms of 50 μl . amounts of the eluates were run in phenol- NH_3 . The results are given in Table 1.

Table 1. *Elution of compound (I) from Zeo-Karb 215 with dilute ammonia solution*

(For details, see text.)

Fractions (serial nos.)	Appearance of filter-paper chromatogram (Phenol- NH_3 -water)
1–8	Faint (I) spot
9	Fairly strong (I) spot
10–12	(I) spot about 10 times as strong as that in fraction 9. Faint pink spot at origin
13–17	(I) spot as in preceding fractions. Faint purple streaks before and after (I)
18	(I) spot about half as strong as that of fraction 9

Fractions 1–7 were discarded. Fractions 8–17 were evaporated to dryness over H_2SO_4 in a vacuum desiccator. 190 mg. of residue were obtained which yielded 120 mg. of material in flat colourless crystals after two crystallizations from aqueous ethanol. (Found: C, 46.0; H, 6.5; N (Dumas), 17.3. $\text{C}_6\text{H}_{10}\text{O}_3\text{N}_2$ requires C, 45.6; H, 6.3; N, 17.7%.) When heated in a sealed melting-point tube at the rate of about 3°/min., signs of decomposition appeared at 173° and increased until 182° had been reached. No melting or further change occurred up to 260°. The tube was cut open at both ends, and the vapour inside blown over the surface of a moist litmus paper which then gave a strong alkaline reaction.

(I) dissolved very readily in water, and the solution gave a slightly acidic reaction with bromothymol blue.

Estimation of amide nitrogen. The microdiffusion method of Dekker, Stone & Fruton (1949) was used, adapted to a small diffusion unit made of paraffin wax.

A solution (50 μl .) made by dissolving 2.223 mg. of (I) in 1 ml. of water was placed in one depression in the wax. 2N- NaOH (50 μl .) was placed in a second depression, and 3N- H_2SO_4 (100 μl .) in a third. The unit was covered by a glass plate and sealed by melting a little wax at the edges of the glass. The alkali was then shaken into the test solution, and the unit left at room temperature for 30 hr. The acid was removed to a test tube, and the alkaline residue digested with nitrogen-free H_2SO_4 and a selenium catalyst. Nitrogen in the mixtures and in a digest of the original solution was estimated colorimetrically after nesslerization. (Found: diffused N, 9.3; residual N, 9.1; total N, 18.2%.)

Following Borsook & Dubnoff (1939), who determined amide N by hydrolysis in N-acid, 1 ml. of solution as used in

the preceding experiment was heated for 3 hr. with 0.5 ml. 3N-H₂SO₄ at 100°. NH₃-N in the hydrolysate, as determined by nesslerization, was equivalent to 8.7% of (I).

Treatment with ozone. 5 mg. of (I) dissolved in water were treated with ozone. When the solution was concentrated in a vacuum desiccator, extensive decomposition occurred with the formation of carbonaceous matter. The main ninhydrin-reacting component gave a purple spot which coincided in position on paper chromatograms with that due to aspartic acid. It could not be separated from added aspartic acid on two-dimensional or long-developed one-dimensional chromatograms in aqueous phenol-NH₃ and *n*-butanol-acetic acid (Partridge, 1948).

The isolation of compound (II). The tops of the young plants from which the exudates had been obtained were broken up in water in a blender and then incubated overnight at 37° in the presence of a little thymol. The autolysates were filtered through muslin and stored in the refrigerator. The autolysates were pooled, charcoal was added and the mixture stirred for 30 min. at room temperature and filtered. The final volume was about 10 l.

The liquid was passed through a column of Zeo-Karb 215, which adsorbed all the ninhydrin-reacting substances. The column was washed with several litres of distilled water, and then a solution of 0.15N-NH₃ was run through. The effluent was evaporated under reduced pressure until the volume was 1.8 l., and then extracted several times with ether to remove fat. Dissolved ether was removed by aeration, and the liquid again treated with charcoal at room temperature and filtered.

A column of De-Acidite B was prepared (Partridge & Brimley, 1949) measuring 40 × 2.4 cm. The liquid was run through at the rate of 5–6 ml./min., and the effluent collected in fractions of approx. 250 ml.

Chromatograms showed that the column had removed the acidic components (aspartic and glutamic acids and compound II) from the first 1.1 l. of liquid. The later fractions (about 650 ml.) contained the acidic as well as other amino-acids.

The column was washed with 1.8 l. of 0.1N-acetic acid followed by 500 ml. of 0.25N-HCl, at the rate of about 100 ml./hr. Fractions were collected at 50–60 min. intervals.

Chromatograms of fractions 1–6 showed glutamic acid and only traces of (II), so they were discarded. Pooled fractions were evaporated by distillation at reduced pressure, and residual water was removed by drying over NaOH in a vacuum desiccator.

Fractional crystallization of the products from water yielded 1.063 g. of crystals which gave only spot (II) on chromatograms, and a number of samples of total weight 2.680 g. which were found to contain slight traces of glutamic acid. The former batch was used for analytical and other quantitative work. The other samples were used for qualitative examinations, and, after further crystallizations, for the preparation of derivatives.

Some properties of compound (II). When heated in a melting-point tube, decomposition commenced at 196°, after which the substance changed in colour through yellow to dark brown at 325°. With more rapid heating (i.e. greater than 2°/min.) the first change occurred at 204°, when the surface of the substance became 'moist'.

The acid had a low solubility in water. No optical activity was detected when 96.3 mg. were made neutral to phenolphthalein with NaOH in water (final vol. 1.82 ml.) and observed in a 1 dm. polarimeter tube. (Found: C, 44.1; H, 5.7; N, 8.6; amino N (Van Slyke nitrous acid method,

4 min. reaction), 8.4. C₆H₉O₄N requires C, 45.2; H, 5.7; N, 8.8%.) When 164 mg. of (II) was titrated with 0.735N-NaOH to a faint pink to phenolphthalein, the equiv. wt. was found to be 158 (formula wt. 159). CO₂ was evolved when (II) was dissolved in 10% (w/v) Na₂CO₃ solution.

Treatment with ozone. 100 mg. of (II) was suspended in 7 ml. of water and ozonized oxygen was passed through for 45 min. The product of the treatment was at first a clear solution, but extensive decomposition occurred when it was allowed to stand overnight. The main ninhydrin-reacting component found on chromatograms coincided in position with aspartic acid.

After removal of the charred material by filtration the solution was boiled with CuCO₃. The excess CuCO₃ was removed by filtration, and, on cooling, a copper compound of low solubility in water crystallized out. Copper was removed from this by H₂S, and 11.1 mg. of a white crystalline solid were obtained by crystallization from aqueous ethanol. This was shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein *et al.* 1947). The product of exhaustive methylation, isolated from the reaction mixture with ether by means of a small continuous extraction apparatus, melted at 295° in a sealed tube. The melting point was unchanged when the product was mixed with an authentic sample of fumaric acid.

Oxidation with permanganate. KMnO₄ solution (1%, w/v) was added to about 1 ml. of a saturated solution of (II), until there was a slight permanent pink colour. This colour was discharged by adding 1 drop more of the solution of (II). The mixture was filtered and electrolytically desalted (Consden, Gordon & Martin, 1947).

Chromatograms run with aqueous *n*-butanol-acetic acid mixture showed a prominent spot which coincided in position with aspartic acid. Two other spots were visible: a well marked spot of lower *R_F*, and a faint spot of higher *R_F* than the aspartic acid.

In another experiment 96 mg. of (II) were dissolved in 4 ml. 10% (w/v) Na₂CO₃ solution. Finely powdered KMnO₄ was added until a slight excess could be detected. A few mg. more of (II) were then added. The mixture was warmed to about 60° for a few minutes, when coagulation of MnO₂ commenced. It was then filtered, and the precipitate washed. A portion of the filtrate was desalted and chromatograms showed a single spot coincident in position with aspartic acid. However, the latter could not be isolated as the Cu salt, presumably because of the high concentration of electrolytes in the mixture. Later, oxalic acid was isolated from residues from this experiment through the Ca salt (melting point of the di-*p*-toluidide 264°, unchanged when mixed with an authentic sample).

Oxidation with cold aqueous permanganate was studied in more detail later.

Reduction of compound (II) to compound (III) and investigation of the product. In one experiment, 28.9 ml. of H₂ were absorbed by 180 mg. of (II) suspended in 10 ml. water with 50 mg. palladium-on-carbon catalyst. (The theoretical amount for a reaction C₆H₉O₄N → C₆H₁₁O₄N under the same experimental conditions was 27.8 ml.)

After removal of the catalyst, the solution was evaporated in a vacuum desiccator. Solid separated when 1–2 ml. of solution remained. This was dissolved by addition of the minimum of water. Acetone was added and the mixture left overnight in the refrigerator. 154 mg. of product were obtained after filtration. (Found: C, 44.7; H, 6.8; N, 9.1. C₆H₁₁O₄N requires C, 44.7; H, 6.8; N, 8.7%.) Equiv. wt. by

titration with 0.103N-NaOH was 159 (equiv. wt. of monobasic $C_6H_{11}O_4N$, 161). Melting points varied from 154–165°, apparently depending on the rate of heating and the amount of material used.

2–3 mg. of (III) were shaken with conc. H_2SO_4 and excess of N_2H in $CHCl_3$ at 43–46° (Adamson, 1939). A sample of the reaction product was desalted and on paper chromatography the main ninhydrin-reacting component of the solution had a higher R_F value than ornithine in aqueous *n*-butanol-acetic acid mixture, being clearly separated from added ornithine.

Synthesis of γ -methylglutamic acid. Preliminary experiments were based on the glutamic acid synthesis of Marvel & Stoddard (1938), methyl methacrylate (containing quinol to inhibit polymerization) being used in place of the methyl acrylate. Methyl methacrylate and diethyl phthalimidomalonate were refluxed in absolute ethanol in which small amounts of sodium had been dissolved. Volatile components were removed after different periods of refluxing, and samples of the residues were hydrolysed overnight with 20% (w/v) HCl in sealed tubes at 105°. Chromatograms run in aqueous *n*-butanol-acetic acid showed the presence of glycine (presumably from free diethyl phthalimidomalonate) and another component coincident in position with compound (III) and presumably γ -methylglutamic acid. The glycine and phthalic acid also present could not be separated from this by crystallization from aqueous ethanol or aqueous acetone. A copper salt of low solubility in water was obtained from the hydrolysis product, which, after removal of the copper by H_2S , was found to yield only a trace of glycine on the chromatograms run in butanol-acetic acid solvent, together with an intense spot coincident in position with (III). 1.83 g. of the copper salt were obtained from 23.8 g. of diethyl phthalimidomalonate. However, preliminary experiments indicated that better results could be obtained by a different procedure.

Snyder *et al.* (1945) synthesized DL-glutamic acid from methyl acrylate and diethyl acetaminomalonate. In the experiment to be described, the conditions were similar to those in the above-mentioned synthesis except that sulphur was added to inhibit polymerization of methyl methacrylate and that an intermediate was isolated and recrystallized before hydrolysis.

Sodium (120 mg., 0.0052 g. atom) was dissolved in approx. 20 ml. ethanol in a 200 ml. flask. 100 mg. sulphur and 10.85 g. (0.05 mole) ethyl acetaminomalonate were added. An attachment bearing a mechanical stirrer, a reflux condenser, and a dropping funnel was fixed to the flask. The contents were refluxed and 10 g. (0.1 mole) of methyl methacrylate were added in drops during 4 hr. Refluxing was continued for a further hour.

The reaction mixture yielded 7.34 g. of colourless crystals after three crystallizations from ethanol. Melting point 109.5°, unchanged on recrystallization. (Found: N (Kjeldahl), 5.7, 5.8%.) This substance was not, apparently, the primary condensation product of methyl methacrylate and ethyl acetaminomalonate, the N content of which would be 4.6%. A small sample was hydrolysed overnight by 20% (w/v) HCl in a sealed tube at 105°, and chromatograms run with *n*-butanol-acetic acid showed a spot coincident in position with (III), and no glycine. 2.78 g. of the intermediate was hydrolysed by refluxing in 20% (w/v) HCl, more HCl being added from time to time to replace the acid which had been used in the reaction.

The hydrolysate was evaporated almost to dryness at reduced pressure. The process was repeated three times after addition of 5 ml. amounts of water. The product was then dissolved in 50 ml. of water. Residual HCl was removed by two extractions of the solution with excess of a 5% (w/v) solution of trioctylamine in $CHCl_3$ (Lester Smith & Page, 1948). The aqueous layer was washed twice with $CHCl_3$ and evaporated in a vacuum desiccator until solid appeared. A further batch of the intermediate (3.62 g.) was hydrolysed and the acid-free product combined with that from the first batch. The crude product was dissolved in the minimum of water. Acetone was added, and the mixture cooled in crushed ice and left overnight in the refrigerator. The resulting solid was filtered off, dissolved in the minimum amount of water, and again treated with acetone, cooled and left overnight in the refrigerator. 2.82 g. crystalline solid were obtained (45% yield, based on the diethyl acetaminomalonate). (Found: C, 44.9; H, 6.8; N, 8.5. $C_6H_{11}O_4N$ requires C, 44.7; H, 6.8; N, 8.7%.)

Oxidative degradation of acid (II). 1.4 ml. of 0.735N-NaOH were added to 161 mg. of acid (II). The solution thus obtained gave a light-green colour with bromothymol blue. A solution of 158 mg. of $KMnO_4$ in 10 ml. water was added in drops over 45 min. The reaction was carried out in a cold room and the temperature of the mixture maintained at 5–6°. After the reaction, the mixture was allowed to stand for 30 min. at laboratory temperature. It was then warmed to 30–35° in a water bath for a few minutes to coagulate the MnO_2 . The precipitate was removed by centrifugation and washed with four lots of water (total vol. 10 ml.). A sample (0.5 ml.) was removed.

A faint turbidity developed when aqueous dimedone solution was added to part of the sample. The remainder of the sample was desalted. Chromatograms run with phenol- NH_3 and *n*-butanol-acetic acid solvents showed that the main ninhydrin-reacting component had a lower R_F than aspartic acid. Smaller amounts of aspartic acid, compound (II) and compound (III) were also present. (The compound (III) had probably been formed by reduction of compound (II) in the desalter.)

0.75 g. of $HIO_4 \cdot 2H_2O$ was added to the combined supernatants. A slight red precipitate formed. This was removed after 1 hr. by centrifugation and the mixture was allowed to stand for a further 30 min. CO_2 was slowly evolved during the periodate reaction. Periodate was precipitated as the Ba salt by addition of $Ba(OH)_2$ in slight excess. The precipitate was washed on the centrifuge, and the combined supernatants made slightly acid with H_2SO_4 and filtered.

The filtrate was distilled and the distillate collected in an aqueous solution containing approx. 260 mg. of dimedone. 71 mg. of a dimedone derivative were obtained. Its melting point was 189°, unchanged when mixed with an authentic sample of the formaldehyde derivative (yield corresponding to 25% of formaldehyde, mol. basis).

A sample of residue from the distillation was desalted. Chromatograms showed a heavy spot corresponding in its position with aspartic acid. The spot could not be separated from that due to added aspartic acid on further chromatograms run in phenol- NH_3 , *n*-butanol-acetic acid, or collidine-lutidine solvents. A desalted sample of the residue from the distillation was analysed by a quantitative chromatographic method (Fowden, 1951). The amount of amino N in the spot referred to above was found to correspond to a yield of 32% (mol. basis) from the amount in the original substance. A similar experiment gave 27% of formaldehyde.

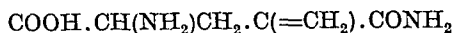
377 mg. of (II) were oxidized in a third experiment. 370 mg. of KMnO_4 and 1.3 g. of $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$ were used. After the treatment with $\text{Ba}(\text{OH})_2$, 330 mg. of dimedone dissolved in hot water were added. The mixture was left overnight at room temperature, and the resulting precipitate filtered off and washed with cold water. 301 mg. of formaldehyde derivative were obtained (mixed m.p. 189° , yield 41%). The filtrate was evaporated to small bulk, filtered, and treated with dimethyl sulphate and 33% (w/v) NaOH (Braunstein *et al.* 1947). 81 mg. of fumaric acid (29% yield) were obtained after continuous ether extraction (m.p. 292° in a sealed tube; 295° after crystallization from water, unchanged on admixture of an authentic sample).

DISCUSSION

The oxidative degradation of (II) and the synthesis of its reduced form have provided evidence on the basis of which the provisional structure



(γ -methyleneglutamic acid) is proposed. The identification of aspartic acid by paper chromatography after treatment of the amide with ozone indicates that the amide is



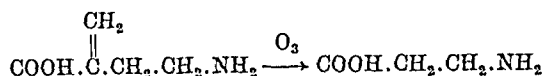
(γ -methyleneglutamine).

Steward & Thompson (1950) presented a synopsis of the results of paper-chromatographic examination of ethanol extracts of various plant materials. They suggested that amides may occur among a group of acid-labile substances detected. However, although asparagine was isolated by Vauquelin & Robiquet (1806) and glutamine by Schulze & Bossard (1883), no similar amino-acid amide has been isolated before the present investigation.

The detection and isolation of the new amino-acid amide (tentatively recognized as γ -methyleneglutamine) and the corresponding acid were made possible by the techniques of paper chromatography. Among other favourable circumstances were: the high concentration of the amide in the exudates, the distinctive ninhydrin reaction of the amide and acid, the low solubility of the acid in water, and the fact that autolysis caused only partial deamidation of asparagine and glutamine, but apparently almost complete deamidation of γ -methyleneglutamine.

The autolysate from which γ -methyleneglutamic acid was isolated contained a substance (IV) which gave a yellow-brown spot at R_f 0.85 in phenol-ammonia. Traces of the same substance were found in the end residues from the fractional crystallizations of the acid, along with a number of other amino-acids. (It is likely that non-acidic amino-acids were retained to some extent on the De-Acidite B and were eluted by the ammonia.) A small quantity of (IV) was isolated from chromatograms of these residues by elution of the appropriate parts of the paper. After treatment with ozone, the product contained a substance which could not be dis-

tinguished from β -alanine on chromatograms run in three different solvents. β -Alanine could be produced from α -methylene- γ -aminobutyric acid:



γ -Aminobutyric acid and β -alanine, the ω -amino-acids related to glutamic and aspartic acids respectively, are now known to be widely distributed among plants. The possible existence of an ω -amino-acid related to γ -methyleneglutamic acid is therefore of considerable interest.

Compounds (I), (II) and (IV) were converted, in the electrolytic cell of the desalter, to substances which gave purple ninhydrin spots. After such treatment, the substance formed from (II) could not be distinguished from compound (III) by paper-chromatographic methods. The R_f 's of the substances formed from (I) and (IV) differed only slightly from those of the original compounds. Apparently compounds (I) and (IV) are also reduced to substances which give a purple colour with ninhydrin. None of the reduced forms has been detected in ethanolic extracts of the plant material.

Application of modern methods to amino-acid analysis and experiments using isotopic tracers are providing increasing evidence that glutamine, asparagine, glutamic acid and aspartic acid are very active metabolites. Thus interesting results may be obtained from further work on the biochemistry of the new type of amide and the corresponding acid described in this paper.

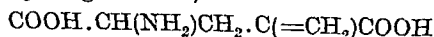
SUMMARY

1. An unsaturated amino-acid amide and the corresponding dicarboxylic amino-acid have been isolated from vegetative material of groundnut plants (*Arachis hypogaea*). The acid showed no optical activity as its sodium salt in water.

2. The provisional structures



(γ -methyleneglutamine) and



(γ -methyleneglutamic acid) have been proposed.

3. The dicarboxylic amino-acid was reduced and the product agreed in properties with a synthetic preparation of γ -methylglutamic acid.

4. The new compounds appear to be normal metabolites of the plants, but the reduced forms were not detected in ethanolic extracts of fresh material.

5. Evidence was obtained that a third unsaturated amino compound was present in the autolysed material.

Sincere thanks are expressed to Prof. B. S. Platt and Prof. W. H. Pearsall for their interest in this work. The elementary analyses were carried out by Mrs V. Shersby and Miss J. Lewis of the National Institute for Medical Research.

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The Carotenoids of the Berries of *Lonicera japonica*

By T. W. GOODWIN

Department of Biochemistry, The University of Liverpool

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Although a great deal is known concerning the distribution and occurrence of carotenoids in fruit (see Karrer & Jucker, 1949; Goodwin, 1952*b*), there are still many gaps in our knowledge; these will have to be filled if a rationale of carotenoid distribution in fruit is eventually to be achieved. Furthermore, studies on the minor carotenoid components of fruit may well throw considerable light on the mode of biogenesis of carotenoids. Only one such investigation on tomatoes has been carried out (Porter & Lincoln, 1950); the results permitted the authors to suggest a working hypothesis for carotenogenesis in this fruit.

No precise information is available on the carotenoids of the berries of the family Caprifoliaceae, although early workers had indicated the presence of lipochromes (carotenoids) in *Lonicera tartarica* (Schimper, 1885), *L. xylosteum* (Schimper, 1885; Molisch, 1896; Kohl, 1902; Nowak & Zellner, 1921); *Sambucus nigra* (Nowak & Zellner, 1921), *Viburnum opulis* and *V. lantana* (Wisselingh, 1914; Nowak & Zellner, 1921; Kryz, 1919). When a small crop of ripe berries of a cultivated climbing honeysuckle (*Lonicera japonica*) became available an investigation was undertaken into the nature of the carotenoids present, with special emphasis on the minor components.

EXPERIMENTAL

Materials. The fully ripened berries were obtained from a garden in north-west Cheshire. Two portions of about 200 g. each were examined separately with identical results.

Extraction and separation of the carotenoids

The fresh berries were ground to a fine powder with Na_2SO_4 and the powder extracted with successive portions of diethyl ether (freshly distilled over reduced iron) until no further colour was extracted. The combined ether extracts were taken to dryness *in vacuo* at room temperature and the lipids saponified by adding to the residue 1 ml. of 60% (w/v) aqueous KOH and 5 ml. of ethanol, mixing and allowing to stand overnight at room temperature. The unsaponifiable matter was extracted into ether as described by Goodwin & Morton (1946) and the ether was removed *in vacuo*; the residue was dissolved in a small volume of light petroleum (b.p. 40–60°) and examined chromatographically. Petroleum, b.p. 40–60°, was used throughout this investigation.

Separation 1. A preliminary separation of the pigment mixture was first carried out on alumina (Spence, Grade 'O') deactivated with methanol (Goodwin, 1952*a*) using light petroleum containing 10% (v/v) of ether as developer. The resulting chromatogram is described in Table 1.

Separation 2. Fraction 1 (Table 1), which eventually percolates through the column, was collected in the filtrate, the mixed solvents removed *in vacuo* and the residue redissolved in light petroleum and chromatographed on activated alumina (Spence, Grade 'O'). Five fractions were obtained as recorded in Table 2.

Separation 3. Zone 6 (Table 1) was eluted with ethanol. The ethanol was removed *in vacuo* at 30° and the residue dissolved in a few drops of ether and made to approx. 10 ml. with light petroleum. This treatment is necessary because this fraction will not dissolve directly in light petroleum. Chromatography was carried out on a column of ZnCO_3 using benzene as developer. Four zones were obtained, two of which eventually moved down the column to be collected

in the filtrate (Table 3). On treatment of the column with benzene containing 5% (v/v) of ethanol, fraction 6C moved off the column and fraction 6D, originally adsorbed at the top, separated into two zones which moved slowly down the column. The faster moving pigment was designated 6DA and the slower moving one 6DB.

Table 1. *The first separation of Lonicera carotenoids on a column of deactivated alumina (Goodwin, 1952a), using light petroleum containing 10% (v/v) ether as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (solvent, light petroleum) (m μ .)
1	Orange, diffuse; greenish blue fluorescence*	—
2	Narrow, orange-khaki	490, 460, 440
3	Pink, slight blue fluorescence*	471, 445
4	Orange	479, 450
5	Trace of khaki	449
6	Orange-red, major fraction: strongly adsorbed	452

* In ultraviolet light.

Table 2. *The separation of fraction 1 (Table 1) on a column of activated alumina (Spence, Grade 'O'), using light petroleum containing 20% (v/v) ether as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (solvent, light petroleum) (m μ .)
1A	Trace of blue-green fluorescence*	348, 367
1B	Lemon-yellow, diffuse, slight blue fluorescence*	399, 425
1C	Colourless, bright green-blue fluorescence*	531, 348, 367
1D	Diffuse-orange: major zone	450, 474
1E	Small, narrow, pale lemon	381, 401, 427†

* In ultraviolet light.

† In ether.

Table 4. *The wavelengths and $E_{1\text{cm}}^{1\%}$ values (in light petroleum) used for quantitative determination of Lonicera carotenoids*

Carotenoid	Wavelength (m μ .)	$E_{1\text{cm}}^{1\%}$	Reference
Phytofluene	348	1200	Porter & Lincoln (1950)
β -Carotene	450	2580	Zscheile <i>et al.</i> (1942)
ζ -Carotene	422	2500	Porter & Lincoln (1950)
γ -Carotene	459	2760*	Zechmeister (1944)
Lycopene	469	3460*	Zechmeister (1944)
Cryptoxanthin	451	2460	Zscheile <i>et al.</i> (1942)
Zeaxanthin	452	2480	Zscheile <i>et al.</i> (1942)
Auroxanthin	425	1780*	Karrer & Jucker (1945)

* Calculated from graphs.

Table 3. *The separation of fraction 6 (Table 1) on a column of ZnCO_3 , using benzene as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (m μ .)
6A	Orange-red, major fraction	464, 491*
6B	Small zone, khaki	438, 463*
6C	Small brick-red zone	453, 479†
6D	Lemon-yellow	429 (broad)†

* Solvent benzene. † Solvent ethanol.

Examination of the fractions

Fractions 2, 3 (Table 1), 1C, 1D, 1E (Table 2), 6A, 6C, 6DB (Table 3). Examination of these fractions, after further chromatographic purification on appropriately activated alumina, indicated that in all probability they were γ -carotene (2), lycopene (3), phytofluene (1C), β -carotene (1D), ζ -carotene (1E), cryptoxanthin (6A), zeaxanthin (6C) and auroxanthin (6DB). In each case, except the last, the berry carotenoid was compared chromatographically and spectroscopically with an authentic and chromatographically homogeneous sample of the corresponding pigment. Samples of all the carotenes were obtained from the fungus *Phycomyces blakesleeanus* (Goodwin, 1952a). Cryptoxanthin and zeaxanthin were obtained from a commercial sample of maize meal.

In the case of fraction 6DB no authentic specimen of auroxanthin was available, but, as demonstrated later, the properties of this pigment (Karrer & Rutschmann, 1942) are so distinctive, that it is virtually impossible to confuse it with any other carotenoid.

Fractions 1A, 1B (Table 2), 6B and 6DA (Table 3). 1A appears to be closely related to phytofluene, whilst 1B is considered to be a carotene not previously described, it is proposed to call it η -carotene. Fraction 4 appears to be very similar to the unidentified pigment reported in human milk (Kon & Mawson, 1950), whilst fractions 5, 6B and 6DA remain unidentified.

Quantitative experiment

In order to obtain information on the relative amounts of the constituent pigments present in the berries, in one experiment the fractions obtained, as described above, were dissolved in known volumes of light petroleum and the extinctions of the solutions measured at the wavelengths of maximal absorption of the pigments concerned. Using the

extinction values of the pigments recorded in Table 4, the amount of each pigment could be calculated. For this present purpose the $E_{1\text{ cm.}}^{1\%}$ (max.) values for the new carotene and the unidentified xanthophylls were assumed to be 2500.

RESULTS

A. The carotenes

The following carotenes were identified unequivocally: phytofluene, β -carotene, ζ -carotene, lycopene and γ -carotene. They were chromatographically indistinguishable from authentic specimens of the corresponding compounds, and the shape and position of their absorption bands in the region 380–500 $m\mu$. (320–400 $m\mu$. in the case of phytofluene) were also identical with those of the known polyenes. Full details have recently been given of the chromatographic and spectral properties of these pigments (Goodwin, 1952a), and further elaboration is unnecessary here.

Fraction 1A (Table 2). This fraction, which only occurred in very small amounts, had an absorption spectrum very similar to that of phytofluene, but appeared to be rather less strongly adsorbed on alumina than this polyene. Insufficient material was available to examine it in any detail, but these preliminary observations do suggest that it is quite distinct from phytofluene.

Fraction 1B (Table 2). This fraction runs down an alumina column as a diffuse dull orange band just ahead of phytofluene and α -carotene. When these chromatographic properties are considered in conjunction with its spectral properties (Fig. 1), there is no doubt that this is a new carotene. The only carotene with similar chromatographic behaviour is ϵ -carotene obtained by Strain & Manning (1943) from the diatoms *Nitzschia closterium* and *Navicula torquatum*. A comparison of the spectrum of ϵ -carotene and that of the *Lonicera* carotene given in Fig. 1 shows that they are quite distinct. The only carotene already reported with an absorption spectrum at all similar to that of the pigment in fraction 1B is ζ -carotene (Porter & Lincoln, 1950). This pigment is, however, easily separable from the *Lonicera* carotene on a chromatogram on alumina (4 parts activated; 1 part deactivated); ζ -carotene is much more strongly adsorbed, for it forms a zone above β -carotene whilst the new carotene travels down the column well in front of β - (and even α -) carotene. Furthermore, ζ -carotene (1 E) and the new carotene occur together in *Lonicera*, and are easily separable. The uniqueness of this carotene being so apparent, it is suggested that it be named η -carotene. This nomenclature follows the recommendations of the 'Union Internationale de Chimie' as drawn up by Karrer (1948). A full investigation into this pigment must await the availability of larger amounts of ripe *Lonicera* berries at a time when it is feasible

to examine them. The discovery of a more potent source of this pigment would also be useful in this connexion, for, as will be seen later (Table 6), η -carotene is only a minor component (1.3%) of the total carotenoids of *Lonicera*.

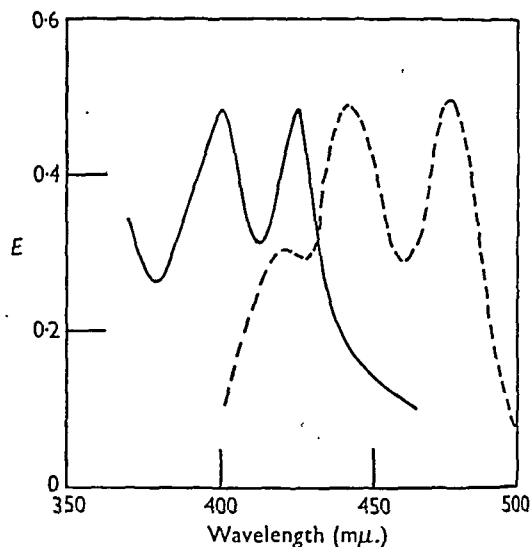


Fig. 1. Absorption spectrum of new carotene (η -carotene) obtained from *Lonicera* berries compared with that of ϵ -carotene from the alga *Navicula torquata*. —, η -carotene in light petroleum (b.p. 40–60°); ----, ϵ -carotene in ethanol (from Strain & Manning, 1943). Note. (i) The change in the position of absorption maxima of carotenes in light petroleum and ethanol is only slight (2–4 $m\mu$.); (ii) the E values are arranged so that E_{max} is the same for both pigments.

Fraction 4. This fraction is epiphasic to 90 and 95% (v/v) aqueous methanol, has an absorption spectrum almost identical with that of β -carotene, and is adsorbed on alumina to very much the same degree as is free vitamin A. It is probable that this is the unidentified pigment found in human blood serum and milk by Kon & Mawson (1950). Their pigment showed absorption bands at 450 and 476 $m\mu$., was adsorbed more strongly than lycopene and could not be separated chromatographically from vitamin A.

Fraction 5. This fraction occurred only in minute traces. It exhibited an absorption spectrum similar to that of β -carotene; insufficient amounts were available for further study.

B. The xanthophylls

Fraction 6A. This fraction was identified as cryptoxanthin; it had the same absorption spectrum (Fig. 2) and the same chromatographic properties as authentic cryptoxanthin. Furthermore, in the partition test, it was not extracted from light petroleum by shaking with 90% (v/v) aqueous methanol; when, however, 95% (v/v) aqueous

methanol was used the pigment was equally distributed in the two phases; this is a characteristic property of cryptoxanthin.

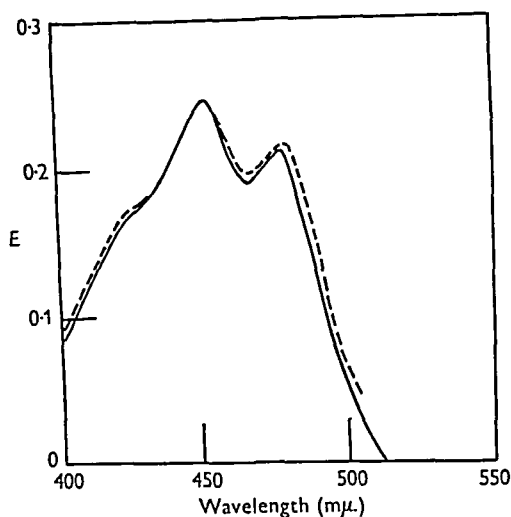


Fig. 2. A comparison of the absorption spectrum of the pigment (zone 6A, Table 3) obtained from *Lonicera* berries with that of authentic cryptoxanthin obtained from maize. The E values are so arranged that E_{\max} is the same for both pigments. ----, *Lonicera* pigment; —, cryptoxanthin. Solvent, light petroleum.

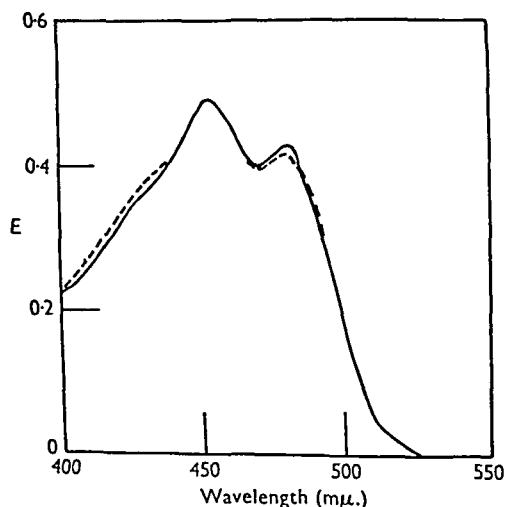


Fig. 3. A comparison of the absorption spectrum of the pigment (zone 6C, Table 3) obtained from *Lonicera* berries with that of authentic zeaxanthin obtained from maize. The E values are so arranged that E_{\max} is the same for both pigments. ----, *Lonicera* pigment; —, zeaxanthin. Solvent, ethanol.

Fraction 6C. A fraction which was completely hypophasic in the partition tests was identified as zeaxanthin by comparison in the usual ways with a specimen of zeaxanthin obtained from maize. A

comparison of the spectrum of the berry pigment with that of authentic zeaxanthin is recorded in Fig. 3.

Fraction 6DB. This fraction appears to be auroxanthin. No authentic auroxanthin was available for comparison, but the very characteristic properties of this pigment leave little, if any, doubt as to its identification. The position and shape of its absorption spectrum is shared by only two other carotenoids, ζ -carotene and aurochrome (and to a lesser degree by η -carotene). A consideration of other properties of these four pigments (Table 5) indicates that the present pigment can only be auroxanthin. A comparison of the spectra of pigment 6DB and that of auroxanthin is recorded in Fig. 4.

Fractions 6B, 6DA. The spectra are recorded in Fig. 4, but could not be identified with any known xanthophylls. The compounds are completely hypophasic. The poor persistence of their spectra suggests that they might be neoxanthophylls (compare the spectral persistence of the neofucoxanthins (Strain, Manning & Hardin, 1943)). Because of the well-known lability of xanthophylls, much further work is necessary before the possibility that these pigments are not merely oxidative artifacts can be completely disproved.

Natural occurrence of the xanthophylls

Partition experiments before saponification showed that the pigments in the crude extract of the berries were almost completely epiphasic using both 90 and 95% (v/v) aqueous methanol, thus indicating that the xanthophylls occurred naturally almost entirely as esters.

The quantitative distribution of the component pigments

The relative amounts of the pigments present in ripe *Lonicera* berries are recorded in Table 6. It will be clearly seen that cryptoxanthin is the predominant pigment.

DISCUSSION

A great deal of the classical work on the isolation of carotenoids for determination of structure has been carried out on fruit of various species (see Karrer & Jucker, 1949, for a complete survey) and, quite naturally, with the emphasis being on isolation of large amounts, minor components were often ignored. Now that, as a result of these investigations, the properties of so many carotenoids are accurately known, it is possible, by utilizing improved chromatographic and spectrographic techniques, to separate and identify carotenoids without the necessity of isolating them in crystalline form. In this way it has recently been shown that tomatoes

Table 5. A comparison of the properties of pigment 6DB (Table 3) with those of auroxanthin, aurochrome, ζ -carotene and η -carotene

Property	Pigment 6DB	Auroxanthin	Aurochrome	ζ -Carotene	η -Carotene
Absorption maxima in light petroleum (m μ .)	400, 425	400, 425	428*	400, 426	399, 425
Partition between light petroleum and 90 % (v/v) aqueous methanol	Hypophasic	Hypophasic	Epiphasic	Epiphasic	Epiphasic
Colour with conc. HCl	Stable blue	Stable blue	Stable blue	None	None
Position on chromatogram	Strongly adsorbed above zeaxanthin	Strongly adsorbed above zeaxanthin	Can be developed on Ca(OH) ₂ with light petroleum; hydroxycarotenoids are not developed with this solvent	Adsorbed above β -carotene but below lycopene	Adsorbed below α -carotene
References	Present work	Karrer & Rutschmann (1942)	Karrer & Jucker (1945, 1949)	Goodwin (1952a) and present work	Present work

* The lower wave band in light petroleum is not recorded by Karrer & Jucker probably for technical reasons. Its existence is, however, obvious from a reference to the curve for aurochrome in CS₂.

contain in addition to lycopene (the major component) very small amounts of a series of polyenes, each differing from the next in the series by four

the synthesis of the fully unsaturated carotenoids (lycopene, β -carotene, etc.). The present work indicates that a very similar series of pigments are present in *Lonicera* berries and this points to a pathway of synthesis similar to that occurring in

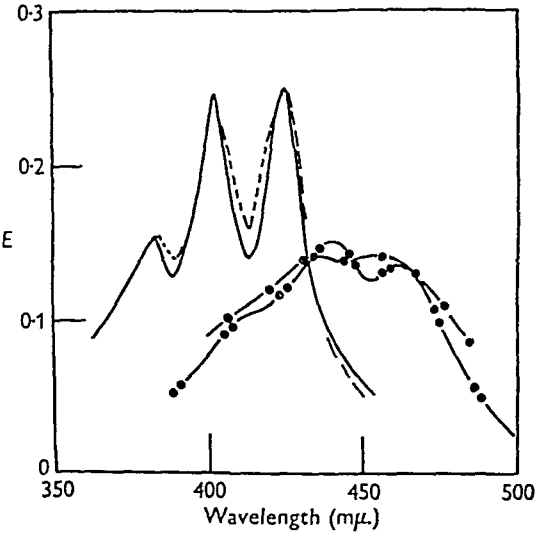


Fig. 4. The absorption spectra of the pigment 6DB (Table 3), authentic auroxanthin, and unidentified xanthophylls 6B and 6DA (Table 3). —, authentic auroxanthin in ethanol (redrawn from Karrer & Rutschmann, 1942); ----, pigment 6DB in ethanol; - · - · -, pigment 6B in benzene; · · · · -, pigment 6DA in ethanol. The *E* values for pigment 6DB and auroxanthin are so arranged that *E*_{max} is the same for both pigments. The *E* values for the other two pigments are those observed in solutions of unknown strength. They bear no relationship to each other or to the *E* values of 6DB and auroxanthin.

hydrogen atoms (Porter & Lincoln, 1950). From this investigation it has been postulated that these pigments represent successive intermediate steps in

Table 6. The quantitative distribution of the polyene components present in *Lonicera* berries, measured as percentages of the total amount of pigments present

Carotenoid	Amount
Phytofluene-like (Fraction 1A, Table 2)	0.45
η -Carotene	1.9
Phytofluene	1.3
β -Carotene	8.95
ζ -Carotene	0.6
γ -Carotene	0.6
Lycopene	2.5
Human milk pigment (Fraction 4, Table 1)	2.9
Unknown (Fraction 5, Table 1)	Trace
Cryptoxanthin	65.2
Unknown (Fraction 6B, Table 3)	1.9
Zeaxanthin	3.5
Unknown (Fraction 6DA, Table 3)	5.2*
Auroxanthin	5.0

* By difference.

tomatoes. As such a series of pigments has never been demonstrated in leaves, this strongly suggests that the route of carotenoid biogenesis in fruit is fundamentally different from that in green leaves.

Recent work has revealed the presence of a very similar series of polyenes in the fungus *Phycomyces blakesleeanus* (Goodwin, 1952a), and earlier work suggests that most of the components of the series also exists in other carotenogenic fungi; the synthetic route in fruit and fungi may thus be very similar. As yet, no such polyene series has been demonstrated in algae, flower petals or bacteria.

Phytofluene, a member of this series, has, however, been observed in a number of flower species (Zechmeister & Sandoval, 1945), and in one bacterium, *Mycobacterium phlei* (Goodwin, 1952a). In the photosynthetic bacterium, *Rhodospirillum rubrum*, however, preliminary experiments suggest that phytofluene is not present (Goodwin & Osman, 1951).

The nature of η -carotene

The position of the absorption spectrum of η -carotene, which is very similar to that of ζ -carotene, suggests that, like ζ -carotene, it contains seven conjugated double bonds. As it is less strongly adsorbed on a column than is ζ -carotene, and as its position on the column is the same relative to β -carotene as that of ζ -carotene is to lycopene, it is possible that η -carotene bears the same structural relationship to β -carotene as ζ -carotene does to lycopene. Thus, it might well be octahydro- β -carotene, with the double bonds symmetrically placed about the centre of the molecule. If either of the double bonds in the β -ionone residues were concerned in the chromophoric system, then one would expect the position of the absorption bands of ζ -carotene to be different from those of η -carotene, in the same way as those of lycopene are different from those of β -carotene.

The small amount of material with a spectrum similar to that of phytofluene, which has been observed to be adsorbed below η -carotene may be, on similar reasoning, dodecahydro- β -carotene, i.e. the β -carotene derivative corresponding to phytofluene, which is probably dodecahydrolycopene (Porter & Lincoln, 1950).

It will be seen from Table 6 that *Lonicera* berries fall into one of the two main categories of carotenoid-containing fruit: those having cryptoxanthin as their major component. The other group tends to accumulate large amounts of lycopene.

The occurrence in the berries of a pigment

(fraction 4, Table 1) which appears to be the 'unidentified pigment' observed in human blood serum and milk by Kon & Mawson (1950) is important because this is the first time it has been reported in plant tissue. Willstaedt & With (1938), who observed a similar pigment in blood serum, considered it to be an 'oxidation product'. This possibility remains, but now the further possibility exists that it occurs in human blood serum and milk as a result of its ingestion in the food.

Two final points of interest may be mentioned:

(1) neither α -carotene nor any of its derivatives occurs in the berries; and (2) this is the first time that auroxanthin has been observed in berries, although it is widespread in flower petals (Karrer & Jucker, 1949).

SUMMARY

1. The following known carotenoids have been found in the ripe berries of the climbing honeysuckle (*Lonicera japonica*): phytofluene, β -carotene, ζ -carotene, γ -carotene, lycopene, cryptoxanthin, zeaxanthin and auroxanthin; an unidentified pigment present in human blood and milk (Kon & Mawson, 1950) also appears to be present.

2. Some spectral and adsorption properties of a new carotene (η -carotene) occurring in the berries are described. Small amounts of a polyene very similar to, but distinct from, phytofluene were also observed.

3. Three pigments occurring in small amounts were not identified; their spectra are recorded.

4. The relative amounts of the pigments present have been determined; cryptoxanthin is the major component. The xanthophylls exist almost exclusively as esters.

5. This work provides additional evidence to support the suggestion that the route of carotenogenesis in fruit is different from that in green leaves.

Thanks are due to Prof. R. A. Morton, F.R.S., for his interest in this work.

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The Application of Counter-Current Distribution to the Separation of Phospholipins

By J. A. LOVERN

Department of Scientific and Industrial Research, Torry Research Station, Aberdeen

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Counter-current distribution between two immiscible solvent phases, as first developed by Craig (1944), offers advantages over both direct and partition chromatography for analytical and preparative work in the field of phospholipins (Lovern, 1949). The only recorded use of the method for such purposes appears to be in a series of papers from one laboratory and deals with vegetable phosphatides (Scholfield, Dutton, Tanner & Cowan, 1948; Scholfield, McGuire & Dutton, 1950; McGuire & Earle, 1951). These workers obtained varying degrees of separation of constituents by distribution between hexane and aqueous methanol or ethanol in a metal apparatus of essentially the original Craig pattern. Various developments in design of apparatus (Lochte & Meyer, 1950; Craig, Hausmann, Ahrens & Harfenist, 1951; Lathe & Ruthven, 1951) have considerably extended the scope of the method, and recently the similar 'cascade distribution' (Kies & Davis, 1951) offers an alternative technique.

The author has studied the applicability of the counter-current technique to the separation of the constituents of the crude glycerophosphatide fraction of the lipids of ox brain, using light petroleum (b.p. 40–60°) and aqueous ethanol as solvents. Preliminary tests, both in a metal apparatus of the original Craig pattern and in a series of separating funnels, suggested that phospholipins are likely to give trouble with apparatus in which all equilibrations and transfers are performed by one operation. They appear to have a considerable effect on the solubility of one solvent in the other. Thus, although the two phases are saturated with each other at the start of the experiment, the relative volumes of the two phases alter appreciably as fractionation proceeds along a series of vessels. This could lead to trouble with mixed phase transfers in an automatic apparatus. Moreover, at certain stages of the separation—usually rather late stages—it occasionally happens that the contents of one vessel will form a very stable emulsion whereas all the rest separate rapidly. This may occur only once or twice in the course of hundreds of transfers, and it can easily be dealt with in a procedure based on individual transfers, but would pass unnoticed in an automatic apparatus. For these reasons, in spite of the great expenditure of time required, the use of a series of

conical flasks has been adopted. They permit the use of any desired volumes and are thus ideal for preparative work. Separating funnels are more easily manipulated, but involve the danger of contamination with stopcock grease. Beroza (1951) prefers bottles to funnels for the same reason.

EXPERIMENTAL

The main experiment was carried out with the total ether-soluble fraction of the crude phospholipins from ox brain. It would, therefore, contain all those lipids based on glycerophosphoric acid (except part of the fully saturated ones, e.g. dipalmityl lecithin), but should be almost free from sphingolipins. Subsidiary trials, for comparative purposes, were made with smaller amounts (about 7 g. each) of crude preparations of (a) phosphatidyl choline, (b) phosphatidyl ethanolamine and (c) phosphatidyl serine. Preparation (a) contained much (b), (b) contained minor amounts of (a), and (c) contained a little (b). Preparations (b) and (c) were made by the method of Folch (1949) from the 'kephalin' of mixed ox brain and spinal cord lipids. All products contained plasmalogen, a constituent which seems to have been ignored by Folch (cf. also Klenk & Böhm, 1951). They were free from acetone-soluble lipids but had not been purified by dialysis.

Ox-brain glycerophosphatide (120 g.) was dissolved in 300 ml. of light petroleum saturated with 85% (v/v) ethanol in the first of a series of twenty conical flasks, the other flasks likewise containing 300 ml. of light petroleum saturated with 85% ethanol. Batches of 300 ml. of 85% ethanol saturated with light petroleum were passed in succession through the train of flasks, with thorough shaking at each stage. Transfer from flask to flask was effected by suction. The successive ethanol extracts were removed as a series of fractions from the end of the series, the process corresponding roughly to elution chromatography. The operation was continued until the weight of lipid in each fraction was very small and only a small proportion of the total lipid remained in the petroleum phase. This gave 62 eluted fractions accounting in all for 93.3% of the total material. The contents of each petroleum fraction were separately recovered, giving a further 20 fractions containing the remaining 6.7% of the original lipid.

The supplementary trials with crude phosphatidyl serine and crude phosphatidyl ethanolamine were run in the same manner, using 100 ml. batches of each solvent phase, in a series of twenty conical flasks, except that only 20 ethanol fractions were run through the train in the former case and, inadvertently, 19 instead of 20 fractions in the latter. The eluted material amounted to 83.3% of the total for phosphatidyl serine and 69% for phosphatidyl ethanol-

amine. The crude phosphatidyl choline was treated differently. It was partitioned between batches of 100 ml. each of light petroleum and 90% (v/v) ethanol in a series of sixteen flasks, but no fractions were run off at the end of the series. Instead, the ethanol phases were allowed to remain successively one flask nearer the first in the series, until all flasks contained both phases. Then the entire contents of each flask were recovered as a single fraction. This corresponds to development of a chromatogram until the solute just reaches the bottom of the column, followed by cutting of the column into sections for extraction.

Grünberg (1938) which, although less reliable than that of Ehrlich, Taylor & Waelsch (1948), involves a simpler technique adequate for observing the progress of separation among a large number of fractions. CHCl_3 was used instead of amyl alcohol as the extractant, since it gives much lower blank values. Comparison was against a standard of pure palmitaldehyde, used as such and not as its glyceryl acetal. Palmitaldehyde was kindly supplied by Mr G. I. Gregory, of the Department of Organic Chemistry, University of Bristol. Phosphorus was determined on certain fractions, using Allen's (1940) method.

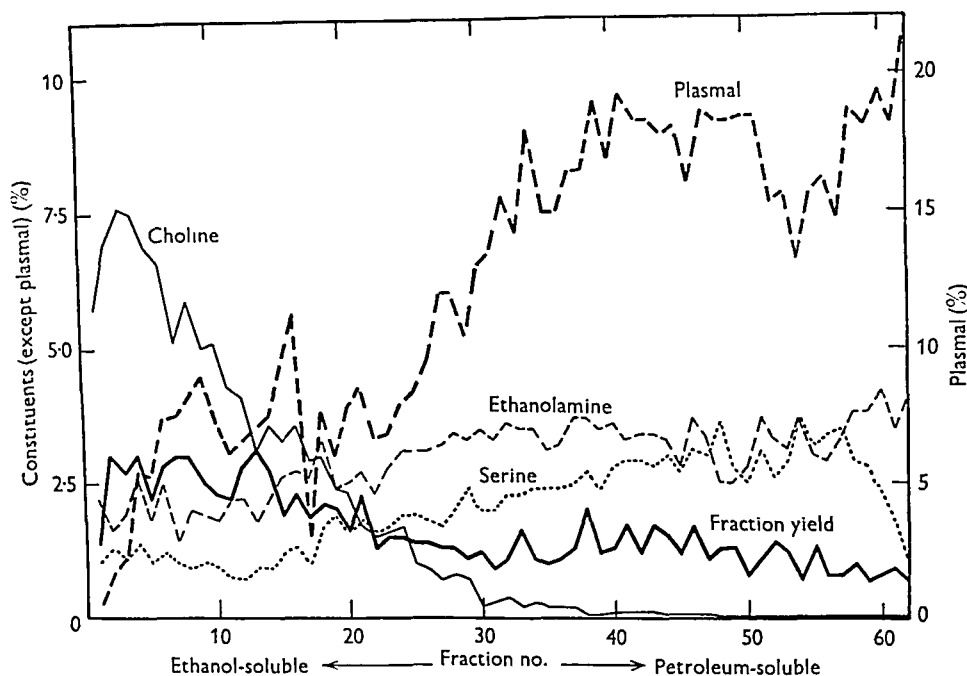


Fig. 1. Ethanol phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

All fractions were examined for choline, ethanolamine, serine and plasmals (higher fatty aldehydes). Inositol was not determined. Paper chromatography (Chargaff, Levine & Green, 1948; Brante, 1948) was used for qualitative examination of the bases present. Choline was determined after refluxing for 2 hr. with ethanolic 0.5N-KOH, followed by addition of water, acidification with HCl, removal of fatty acids with ether and evaporation of the aqueous solution to dryness on the steam can under vacuum, to remove excess HCl. The residue was made up to known volume with water (saturated with CHCl_3 to prevent microbial destruction if any appreciable delay should occur) and the choline estimated by Glick's (1944) method, combined with the use of Beattie's (1936) aqueous reineckate solution and Winzler & Meserve's (1945) ultraviolet absorption technique. Ethanolamine and serine were determined after refluxing for 6 hr. with 6N-HCl, removal of fatty acids with ether, evaporation to dryness and solution in water to known volume as for choline. The ethanolamine and serine content was then determined by a combination of the methods of Artom (1945) and Burmaster (1946), permittit being replaced by the synthetic resin Zeo-Karb 215. Plasmals were determined by the method of Feulgen &

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained for the 62 fractions of the ethanol phase of the ox-brain glycerophosphatides, for yield (percentage of total ethanol-phase lipid) and content of the three nitrogenous components and of plasmals, all expressed as percentages. Fig. 2 shows similar results for the 20 fractions of the petroleum phase, except that serine and ethanolamine have been given as total amino base. This was predominantly ethanolamine. The results obtained on the crude phosphatidyl choline are shown in Fig. 3, those for the ethanol phase of crude phosphatidyl ethanolamine in Fig. 4 and for the petroleum phase of the same fractionation in Fig. 5. Fig. 6 shows the data obtained on the ethanol phase of the crude phosphatidyl serine, most of the petroleum phase fractions being too small for analysis. In Figs. 1 and 4 the plasmal scale is half that for the other components, but in the other diagrams it has proved convenient to use the

same scale for all components. To facilitate comparison of the various Figures, fraction numbering in all cases has been arranged so that number 1 represents the fraction most preferentially soluble in the ethanol phase.

deep orange in colour and only semi-solid at room temperature. Fraction 3 was a little paler and more solid. Fractions 4-7 were yellow and quite firm solids, fractions 8-12 were cream-coloured firm solids, fractions 13 and 14 were yellow soft semi-

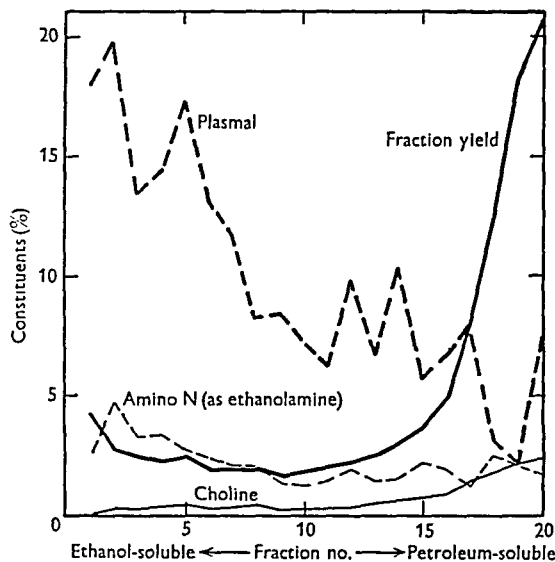


Fig. 2. Petroleum phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

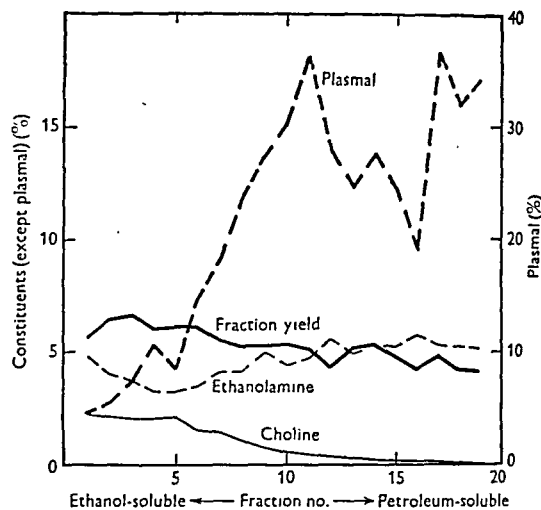


Fig. 4. Ethanol phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

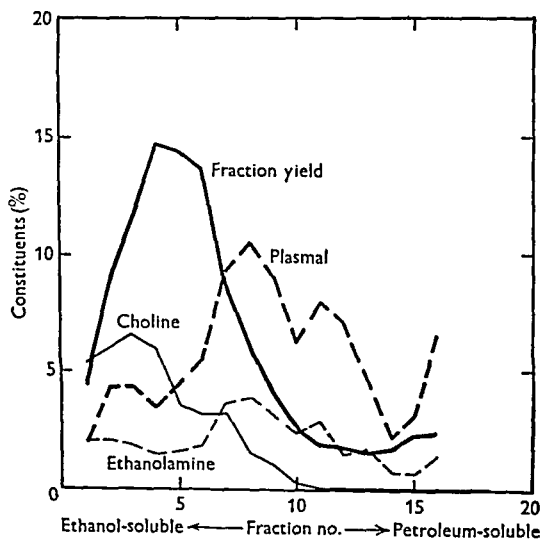


Fig. 3. Combined phases in the distribution of crude phosphatidyl choline between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

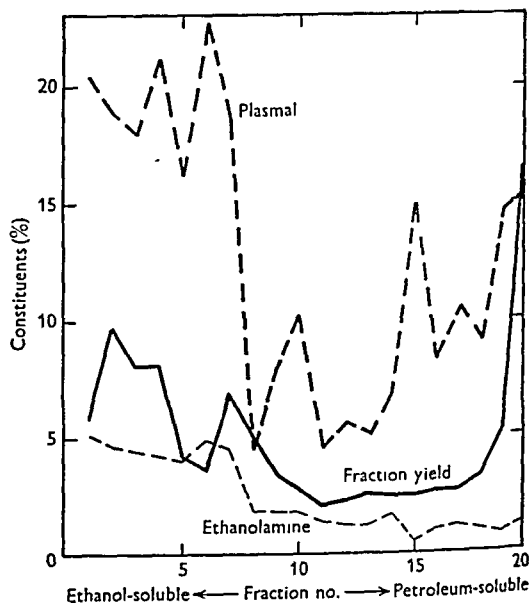


Fig. 5. Petroleum phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Considering first the main experiment, the fraction-yield curve in Fig. 1 indicates a whole series of overlapping fractions and various changes in appearance of the fractions also suggest a complex distribution pattern. Fractions 1 and 2 were

solids, fraction 15 was again cream coloured and somewhat firmer, fractions 16-57 were cream-coloured firm solids and fractions 58-62 were yellowish brown firm solids.

As might be expected from the known solubility properties of the phospholipins, phosphatidyl choline is removed by the ethanol preferentially to most of the other constituents, but the curve indicates the presence of derivatives of considerably different partition coefficients, varying presumably in fatty acid components. Some of these, e.g. the small proportion of the total phosphatidyl choline represented by fractions 30-37, are markedly different from the main fraction represented by fractions 1-7. Phosphatidyl choline is not completely extracted until fraction 49.

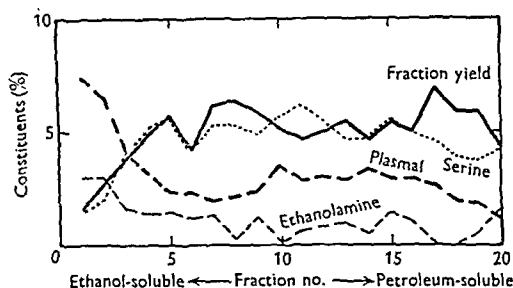


Fig. 6. Ethanol phase in the distribution of crude phosphatidyl serine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Figs. 3 and 4 likewise show the early extraction of phosphatidyl choline by the ethanol. The lipid preparations in question had, of course, already been separated on the basis of relative solubility in ethanol, and as the starting material (mixed brain and spinal cord as against brain only) was also different, the curves would not be expected to duplicate very closely those in Fig. 1. It may be significant that the long tail-off in the extraction of phosphatidyl choline appears in Fig. 4 for the less ethanol-soluble preparation, but is not seen in Fig. 3. On the other hand, Fig. 3 shows evidence of at least two phosphatidyl cholines, one represented by fractions 1-4, and one by fractions 5-7. These may well correspond to fractions 1-12 and 13-18 respectively in Fig. 1.

It might be expected that the curve for ethanolamine would be particularly complex, since this base is present in two lipid classes, phosphatidyl ethanolamine and plasmalogen. In Fig. 1, from fraction 1 to about fraction 22, the ethanolamine curve shows violent fluctuations, with indications of some large main fractions, e.g. fractions 13-22 inclusive. In these first 22 fractions, apart from the earliest ones and, apparently, fraction 17, the ethanolamine is derived from both the above types of phospholipin. A plasmal content of 7.5%, about the mean level of fractions 8-22, would account for an ethanolamine content of about 1.8%, whereas the total ethanolamine content of these fractions is

of the order of 2.5%. The plasmal curve covering these same 22 fractions shows evidence of more than one plasmalogen, presumably involving a different aldehyde. There is considerable discrepancy in the literature as to the range of aldehydes present in brain plasmalogen (Thannhauser, Boncoddio & Schmidt, 1951; Leupold, 1950). At the time the present work was completed it had not been reported that plasmalogens may have a base other than ethanolamine, but some of the results, discussed later, suggest that this must be the case. A very recent paper by Klenk & Böhm (1951) provides evidence for a plasmalogen containing serine instead of ethanolamine. In spite of the relatively inaccurate technique, there are clearly three distinct plasmalogen fractions represented roughly by fractions 1-11, 12-17 and 18-22 respectively.

From fractions 23 to 44 inclusive, the ethanolamine curve shows only small fluctuations, some within the limits of experimental error. Over this same range of fractions, the plasmal curve climbs steadily to a roughly constant level of 17%, which is then maintained from about fractions 42 to 51. Some of the peaks and depressions in the plasmal curve over the fraction range 23-44 can be correlated with similar features in the ethanolamine curve, e.g. the drop in both curves for fractions 35 and 36, but many of the smaller irregularities in the plasmal curve may be due to experimental error. A plasmal level of 17% requires about 4% of ethanolamine, fully as much as the total present. Although the estimation of natural plasmalogen against free palmitaldehyde is not likely to give an accurate figure for the plasmal content of the fractions, its approximate correctness was confirmed by estimation of the acetone-soluble lipid set free when an ether solution of the fraction was emulsified with a solution of mercuric chloride, which rapidly breaks the acetal linkage in plasmalogen (Feulgen, Imhäuser & Behrens, 1929).

From fraction 45 onwards the ethanolamine curve again shows considerable fluctuations with evidence of at least three main components: fractions 45-48, 49-57 and 58-62 respectively. Some fractions show trends in the ethanolamine curve in this range corresponding to similar trends in the plasmal curve, e.g. the very low value for fraction 57, but most of them are entirely unconnected, e.g. the ethanolamine peak at fraction 54 is associated with a pronounced minimum on the plasmal curve. There is, rather, evidence in fractions 45 to at least 57 of the occurrence of phosphatidyl ethanolamines different from those of fractions 1-22, a point referred to again in considering the serine curve. The plasmal curve shows clear evidence of more than one fraction over this later range, the minimum between fractions 51 and 58 being outstanding.

The results on crude phosphatidyl choline (Fig. 3) show a much closer parallelism between the distribution of plasmalogen and ethanolamine. They agree with Fig. 1 in suggesting more than one plasmalogen (fractions 1-4, 5-14, 15 and 16). Again it is in the earlier fractions that the highest ratios of phosphatidyl ethanolamine to plasmalogen are found. At the peak of the plasmal curve there is still a considerable excess of ethanolamine (4%) over that required by the plasmal (about 2.4%), but at fraction 16 the ratio of plasmal to ethanolamine is just right for plasmalogen.

In Fig. 4, representing a relatively less ethanol-soluble lipid than Fig. 3, there is evidence of two plasmalogens, the plasmal curve corresponding fairly well to the later part of that in Fig. 1 (from fraction 17 onwards). The ethanolamine curve does not run parallel with it and, as in the other cases, ethanolamine differs from choline in not showing any tendency to elimination as fractionation proceeds. Fig. 4 emphasizes that lipids rich in ethanolamine tend to be rich in plasmalogen, but it also provides direct evidence that not all this plasmal can be present in ethanolamine-containing plasmalogen. Some of the fractions contain about 36% of plasmal, requiring about 8.6% of ethanolamine, whereas only 5% is present. At fraction 16 the ratio of the two substances is about right for the accepted plasmalogen structure. In the earlier fractions there is a great excess of ethanolamine, obviously present as phosphatidyl ethanolamine.

In the partition of the crude phosphatidyl ethanolamine the process was not carried near to completion, and Fig. 4 should be compared with the early part of Fig. 5. For fractions 1-7 (Fig. 5) the plasmal content of about 20% requires about 4.8% of ethanolamine, near to the 4.5% which is present. The later fractions in Fig. 5, however, tell a different story. They contain 10-15% of plasmal, requiring about 3% of ethanolamine, whereas only about 1% is found.

The crude phosphatidyl serine, Fig. 6, contains very little plasmalogen, and where ethanolamine is present it is more than adequate to account for the plasmal. But in one or two fractions no ethanolamine at all could be detected, yet plasmal in about the same amount as in the neighbouring fractions was still present.

The serine curve in Figs. 1 and 6 resembles the ethanolamine curve in being extended over the full range of fractions. In Fig. 1 it shows only minor fluctuations from fractions 1 to 17, though with a definite minimum in fractions 11 and 12, after which there is a sudden increase followed by a fairly general rise with minor fluctuations to fraction 44. From here until fraction 57 the serine curve exhibits a series of abrupt rises and falls rivalling those of ethanolamine over the same fraction range, the

two curves being sometimes in phase and sometimes out of phase. In general, the similar behaviour of phosphatidyl ethanolamine and phosphatidyl serine is not surprising, in view of the extreme difficulty in separating these lipids by the classical methods. The curves in Fig. 1 suggest that they have a similar assortment of fatty acids and that both contain representatives, appearing in fractions 45 onwards, considerably different in fatty acid composition from those appearing in the earlier fractions. Klenk & Böhm (1951) emphasize the complex range of fatty acids present in both phosphatidyl ethanolamine and phosphatidyl serine, but report great differences in this respect between the two lipids. However, they mention that intermediate fractions, discarded in the preparation of purified fractions, may have influenced this. It was noted that fractions 45-62 (especially fractions 60-62) were much more slowly broken down by acid hydrolysis than were all the earlier fractions. The generally slower rise of the serine curve until it meets the ethanolamine curve at fraction 45 is in line with the relative solubilities of the two lipids in ethanol (Folch, 1948).

The serine curve in Fig. 6 exhibits a steep initial rise instead of the long slow rise in Fig. 1. There are several peaks and depressions, the agreement between Figs. 1 and 6 showing that there must be a number of phosphatidyl serines, differing in their fatty acid components.

In all cases the combined derivatives of choline, ethanolamine and serine can account for only a portion of the total fraction, e.g. ranging for Fig. 1 from 48 to 89%, with a value of about 60% for the majority of the fractions. Phosphorus determinations, made on every fifth fraction in Fig. 1, ranged from 2.0 to 3.7%, with most in the range 2.5-3.5%, thus corresponding quite well with the values obtained from the nitrogenous derivatives. Part of the balance in some fractions may consist of inositol lipids, but a much greater factor seems to be the presence of lipids of the wax ester type. All the fractions in Fig. 1 yield appreciable amounts (ranging from 3 to 15%, with the lowest values in the early fractions) of unsaponifiable matter, by ether extraction of alkaline hydrolysates. According to Feulgen *et al.* (1929) the acetal linkage of plasmalogen is completely resistant to 5 hr. boiling with 5% aqueous sodium hydroxide, and ether extraction of the solution should leave the aldehyde in the aqueous layer as the sodium salt of plasmalogenic acid. In the author's laboratory, where ethanolic 0.5N-potassium hydroxide is routinely used for alkaline hydrolysis, it has been found that small, variable amounts of plasmal are set free in 2 hr. refluxing. Accordingly, unsaponifiable matter has been determined after removal of plasmal with mercuric chloride. The unsaponifiable matter from

all fractions had a similar appearance, being a soft paste at room temperature and a mobile liquid when warm. It had an iodine value ranging from 70 to over 100 in the various fractions. The total product was submitted to fractional distillation at 0.1 mm. pressure, but only a few drops distilled over the range 120–180°, when distillation was stopped. Distillate and residue were recombined and acetylated. The product had a saponification equivalent of 411.3, corresponding approximately to the acetate of a C_{25} monohydric alcohol. There would almost certainly be a mixture of homologues present, probably those of an even number of carbon atoms. Such alcohols, presumably esterified with fatty acids, do not seem to have been reported previously in the crude phospholipin fraction of ox brain.

Turning to Fig. 2, and comparing it with Fig. 5, it can be seen that the greater part of the material recovered from the petroleum phase has remained virtually unmoved by the ethanol. Following the initial fractions containing material closely resembling the final fractions of the ethanol phase, especially marked in the incomplete procedure represented in Fig. 5, there is a long run of very small fractions and then an abrupt rise.

The nature of the last few fractions is obscure. In Fig. 2 appreciable amounts of choline are shown, which cannot be present as phosphatidyl choline. The obvious suggestion would be small amounts of sphingomyelin but these fractions do not have the solubility properties of sphingomyelin. They are firm solids readily soluble in cold ether or light petroleum. They are quite insoluble in ethanol. They also contain appreciable amounts of plasmalogen and ethanolamine. It should be noted that they account for only a very small proportion (less than 0.5%) of the total for the crude glycerophosphatides of ox brain, but considerably more—about 8%—of the crude phosphatidyl ethanolamine of the mixed brain and spinal cord. The very small fractions covering the long flat minimum of the petroleum phase-yield curve are different again, being soft gummy products which become liquid on warming.

It seems clear that such a complex mixture as the total glycerophosphatides of ox brain requires a far

longer fractionation chain than 20 units to effect sharp separation of major constituents, and that even under the best conditions there would be poor separation of some of them, e.g. of serine-containing from ethanolamine-containing lipids, unless other solvent pairs should give better results. However, in Fig. 1, fractions 1–20 contain most of the phosphatidyl choline with relatively small proportions of lipids containing serine and ethanolamine. Fractions 30–45 should give a fraction consisting mainly of plasmalogen and phosphatidyl serine, and fractions 58–62 should contain an even purer plasmalogen. When it is remembered how markedly phospholipins influence the solubility properties of other phospholipins it is not surprising that sharp separation is not achieved by the relatively simple fractionation procedure used in these experiments.

SUMMARY

1. The crude glycerophosphatides of ox brain, and crude preparations of phosphatidyl choline, phosphatidylethanolamine and phosphatidylserine, have been partitioned between aqueous ethanol and light petroleum, employing the technique of counter-current distribution.

2. The results suggest a useful degree of separation of phosphatidyl choline from similar lipids containing ethanolamine and serine and of plasmalogen from lipids other than phosphatidyl serine. Separation of phosphatidyl ethanolamine from phosphatidyl serine is poor.

3. There is evidence that the plasmalogens are a complex group which may include derivatives with a base other than ethanolamine, as well as a range of aldehydes. The glycerophosphatides containing choline, ethanolamine and serine all show evidence of multiplicity of fatty acid composition.

4. The crude phospholipins of ox brain contain substances not so far classifiable, including choline-containing lipids different from lecithin and sphingomyelin, appreciable amounts of what appear to be wax esters, and gummy lipids of unknown composition.

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Estimation of Nucleic Acids in Tissue from the Nervous System

By J. E. LOGAN, W. A. MANNELL AND R. J. ROSSITER

Department of Biochemistry, University of Western Ontario, London, Canada

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The starting point of this investigation was the report of Davidson & Waymouth (1944) that white matter of the brain of the sheep contained a higher concentration of protein-bound phosphorus, i.e. phosphorus remaining after removal of lipid phosphorus and acid-soluble phosphorus, than grey matter. If most of this protein-bound phosphorus were present as nucleic acid, there remained the possibility that nucleic acid might be an important constituent of the myelin sheath of a mammalian nerve fibre. Since no nucleic acid can be detected in the myelin sheath by the usual histochemical means, it seemed worth while to investigate the matter further. Attempts were made to estimate the concentration of deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) in different parts of the nervous system by two methods at present widely used, that of Schmidt & Thannhauser (1945) and that of Schneider (1945). Although recovery experiments showed that added DNA, PNA, or mixtures of the two, could be recovered quantitatively from brain extracts by either method, it soon became apparent that the two methods gave widely diverging results when they were applied to white matter and grey matter of brain and to peripheral nerve.

It was decided, therefore to investigate the validity of these methods for tissue from the nervous system. The conflicting results are due, in part, to the presence of considerable amounts of a phosphorus-containing compound in brain and spinal cord, particularly in the myelin-rich white matter. The phosphorus of this compound is not removed by ice-cold 10% (w/v) trichloroacetic acid (TCA) nor is it removed by the usual ethanol-ether treatment for the extraction of lipids. This substance is probably the same as the inositol-containing

trypsin-resistant lipid-protein complex isolated from white matter by Folch & Le Baron (1951). Dr J. Folch, McLean Hospital, Waverley, Mass., in experiments as yet unpublished, has obtained results similar to those reported here.

In addition, it is shown that TCA extracts of brain and nerve tissue, prepared as described by Schneider (1945), contain chromogenic material that interferes with the colour reactions of Dische (1930) for DNA and Mejbaum (1939) for PNA. Folch (1951) suggests that this interference may be caused by traces of the complex lipid substance, strandin, isolated from brain by Folch, Arsove & Meath (1951). A preliminary account of these experiments has already appeared (Rossiter, Logan & Mannell, 1951).

METHODS

Standards

The same DNA and PNA standards were employed throughout this work. Results obtained for each of the methods are comparable inasmuch as they are all given in terms of the P content of these standards. The DNA standard was a preparation of calf sodium thymonucleate prepared by the

Table 1. *Absorption characteristics and phosphorus content of standard preparations of DNA and PNA*

	$\lambda_{\max.}$ (m μ .)	ϵ (P) at $\lambda_{\max.}$	$\lambda_{\max.}$ (m μ .)	ϵ (P) at $\lambda_{\min.}$	P (%)
DNA	259	7300	230	2900	8.3
PNA	258	9300	228	3500	8.5

method of Mirsky & Pollister (1942), and kindly provided by Dr G. C. Butler, Department of Biochemistry, University of Toronto. The PNA standard was a preparation of yeast sodium ribonucleate selected from a number of commercial preparations examined. Table 1 shows that the absorption

characteristics and P content of the standards are in good agreement with the published figures for DNA and PNA (Stimson & Reuter, 1945; Di Carlo & Schultz, 1948; Blout & Fields, 1949; Chargaff *et al.* 1949, 1950).

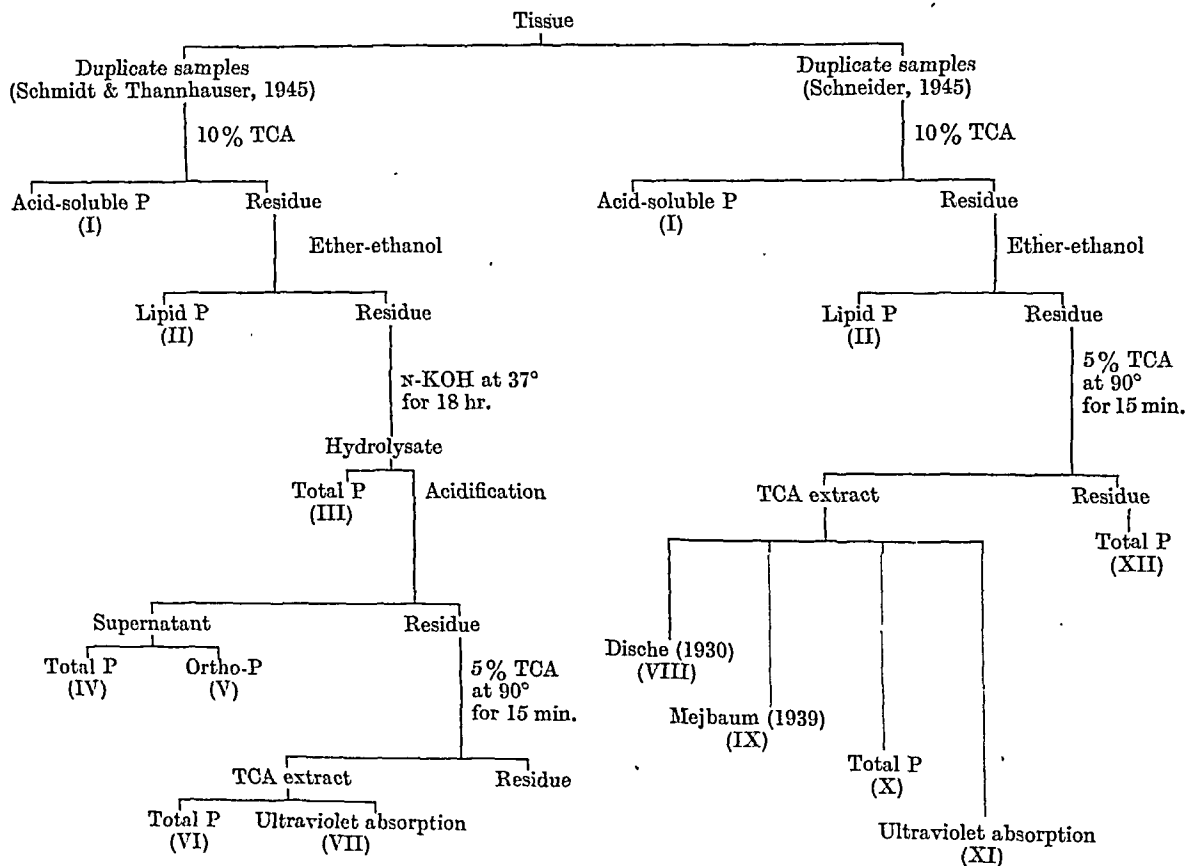
The symbol $\epsilon(P)$ is used for the atomic extinction coefficient with respect to P as defined by Chargaff & Zamenhof (1948), i.e. $\epsilon(P) = \frac{30.98 E}{cl}$, where E is the extinction ($\log I_0/I$), c is the concentration of P in g./l. and l is the thickness of the absorbing layer in cm. The value of $\epsilon(P)$ at λ_{\max} is a little greater than that given by Chargaff *et al.* (1949). The value of $\epsilon(P)$ probably depends upon the degree of polymerization of the sample (Chargaff *et al.* 1950). For samples of thymus DNA, presumably less highly polymerized, Stimson & Reuter (1945) and Blout & Fields (1949) reported much higher values of $\epsilon(P)$.

matter was chiefly corpus callosum. The sciatic nerve of the cats was rapidly cleaned of adherent fatty and epineural connective tissue and the whole nerve frozen and ground to a powder as described for the dog brain.

Analytical methods

For each tissue two pairs of duplicate samples of the frozen powder (100–200 mg.) were transferred to tared centrifuge tubes and the tubes reweighed. After the removal of acid-soluble P (I) and lipid P (II), as described by Schneider (1945), each pair of duplicate samples was submitted to either the Schmidt-Thannhauser or Schneider procedure as outlined in Table 2. One pair was hydrolysed for 18 hr. at 37° in N-KOH. The hydrolysate was divided into two portions, one of which was used for the determination of total P (III) and the other for the precipitation of the

Table 2. Scheme outlining determinations



Preparation of tissue

Estimations were done on the white matter and grey matter of the brains of six dogs and on the sciatic nerves of six cats. The animals were anaesthetized with nembutal, bled, and the brain or sciatic nerve removed as soon as possible after death. Samples of brain tissue (0.5–1 g.) were frozen in liquid N₂ and ground to a fine powder in a mortar. The sample of grey matter consisted of thin shavings of cortex from the cerebral hemispheres. The sample of white

DNA with 5N-HCl and 5% (w/v) TCA. The DNA was removed by centrifuging. One portion of the supernatant was used for the determination of total P (IV) and another for the determination of the ortho-P (V) by the modification of the method of Berenblum & Chain (1938) described by Ernster, Zetterström & Lindberg (1950). The precipitated DNA was extracted with 5% (w/v) TCA for 15 min. at 90°, as described by Schneider (1946). The total P was estimated in one portion of the extract (VI) and the DNA was estimated by ultraviolet absorption (VII) in another. Details

of this method are given in the Appendix. The residue remaining after the extraction of the DNA contained a negligibly small concentration of P.

The other pair of duplicate samples from which the acid-soluble P and lipid P had been removed was treated with 5% (w/v) TCA for 15 min. at 90°, as described by Schneider (1945). Portions of the TCA extract were used for the determination of DNA by the diphenylamine reaction of Dische (1930) (VIII), for the determination of PNA by the orcinol reaction of Meibbaum (1939) (IX), for the determination of total P (X), and for the determination of total nucleic acid by the method of ultraviolet absorption described in the Appendix (XI). A total P estimation was also done on the residue remaining after the TCA extraction (XII).

All fractions were made up in volumetric flasks, and portions containing 10–20 µg. P were taken for each P determination. Total P was estimated by the method of King (1932). The colour was read in a Coleman Universal spectrophotometer at 650 mµ. The diphenylamine and

for the lipid P of white matter and grey matter of dog brain and cat sciatic nerve are of the same order as those reported for similar mammalian tissues (see Johnson, McNabb & Rossiter, 1948, 1949, for references). It will be noted that, as originally pointed out by Davidson & Waymouth (1944), the concentration of protein-bound P in white matter exceeds that in grey matter.

Table 4 compares the concentration of total nucleic acid determined by the Schmidt-Thannhauser method with that determined in the Schneider TCA extract by (a) the colour reactions, (b) the total P, and (c) the ultraviolet-absorption method. The values obtained by the Schmidt-Thannhauser method are all greater than those for the total P in the TCA extract and these, in turn, are greater than the values for the ultraviolet-absorption method. The greatest difference is in white

Table 3. *Distribution of phosphorus compounds in tissue from the nervous system*

(Mean \pm S.E.M. of six determinations, expressed as mg. P/100 g. wet tissue.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve
Acid-soluble P	I	74.5 \pm 4.2	79.5 \pm 3.1	52.0 \pm 2.1
Lipid P	II	333 \pm 14	157 \pm 6	308 \pm 23
Protein-bound P	III	33.9 \pm 2.4	24.6 \pm 1.1	22.3 \pm 1.0
Total P	I + II + III	441 \pm 19	261 \pm 10	382 \pm 26

Table 4. *Total nucleic acid of tissue from nervous system*

(mg. P/100 g. wet tissue. Mean of six experiments \pm S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	III – V	31.3 \pm 1.7	23.8 \pm 0.6	20.1 \pm 1.0	DNA + PNA + 'inositide P'
Schneider (1945), colour reactions	VIII + IX	21.1 \pm 2.5	25.4 \pm 1.2	14.9 \pm 1.1	DNA + PNA + ?
Schneider (1945), phosphorus	X	18.8 \pm 2.5	16.5 \pm 0.3	14.0 \pm 1.5	DNA + PNA + part of 'inositide P'
Ultraviolet absorption	XI	10.7 \pm 0.5	15.2 \pm 0.3	10.2 \pm 0.5	DNA + PNA

orcinol reactions were performed as described by Schneider (1945), the final readings being made at 600 and 655 mµ. respectively. Results are all presented in terms of the P content of the standard solutions.

RESULTS

Comparison of methods

Table 3 shows the concentration of acid-soluble P, lipid P and protein-bound P in the white matter and grey matter of dog brain and the sciatic nerve of the cat. These values agree in most respects with those reported for the brain and nerve of the monkey by Bodian & Dziewiatkowski (1950), and for the sciatic nerve of the guinea pig by Samuels *et al.* (1951). The concentration of lipid P in white matter is more than twice that in grey matter. The figures

matter and the least in grey matter. The values given by the colour reactions are also considerably higher than those given by the method of ultraviolet absorption.

Tables 5 and 6 give the values obtained for the concentration of DNA and PNA by a number of different methods. For all three tissues there is good agreement between the figures for DNA obtained by the Schmidt-Thannhauser method, the modification of this method described by Schneider (1946), and the ultraviolet-absorption method. The only values that do not agree are those given by the diphenylamine colour reaction of Dische (1930). These are higher for all three tissues.

It was with the PNA that the greatest differences were found (Table 6). The values for the concentration of PNA obtained by the Schmidt-Thannhauser

method are all considerably greater than those obtained by the ultraviolet absorption method. The greatest difference is for white matter and the least is for grey matter. The values obtained by the orcinol colour reaction of Mejbaum (1939) are also slightly greater for all three tissues ($P < 0.01$, in each instance). Table 6 also gives the values of two

extraction (XII), are greater than those found for the 'phosphoprotein' of Schmidt & Thannhauser (1945), i.e. the ortho-P liberated on hydrolysis in *N*-potassium hydroxide at 37° for 18 hr. (V). The difference is again greatest for white matter and least for grey matter, with the sciatic nerve being intermediate.

Table 5. *DNA of tissue from nervous system*(mg. P/100 g. wet tissue. Mean of six experiments \pm S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	III - IV	6.1 \pm 0.2	4.5 \pm 0.2	4.8 \pm 0.2	DNA
Schmidt & Thannhauser (1945), modified by Schneider (1946)	VI	6.7 \pm 0.2	4.7 \pm 0.3	5.5 \pm 0.2	DNA
Schneider (1945), colour reaction of Dische (1930)	VIII	14.2 \pm 2.1	13.1 \pm 1.5	6.6 \pm 0.6	DNA + ?
Ultraviolet absorption	VII	5.8 \pm 0.4	4.9 \pm 0.4	5.4 \pm 0.4	DNA

Table 6. *PNA of tissue from nervous system*(mg. P/100 g. wet tissue. Mean of six experiments \pm S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	IV - V	25.2 \pm 1.6	19.3 \pm 0.8	14.3 \pm 0.8	PNA + 'inositide P'
Schneider (1945), colour reaction of Mejbaum (1939)	IX	6.9 \pm 0.4	12.4 \pm 0.5	7.9 \pm 0.7	PNA + ?
Combination I of Schmidt & Thannhauser (1945), and Schneider (1945)	IV - XII	10.7 \pm 1.5	11.6 \pm 0.7	8.1 \pm 1.0	PNA + part of 'inositide P'
Combination II of Schmidt & Thannhauser (1945), and Schneider (1945)	X - VI	11.0 \pm 2.3	11.1 \pm 0.6	6.8 \pm 0.5	PNA + part of 'inositide P'
Ultraviolet absorption	XI - VII	4.9 \pm 0.2	10.3 \pm 0.4	4.7 \pm 0.5	PNA

Table 7. 'Phosphoprotein' of tissue from nervous system

(mg. P/100 g. wet tissue. Mean of six experiments \pm S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
'Phosphoprotein' of Schmidt & Thannhauser (1945)	V	2.6 \pm 0.2	2.4 \pm 0.2	2.2 \pm 0.2	PP ('phosphoprotein', phosphorus)
'Phosphoprotein' of Schneider (1945)	XII	15.3 \pm 1.2	7.7 \pm 0.6	10.2 \pm 0.9	PP + part of 'inositide P'
'Inositide P'	III - (V + XI)	20.6 \pm 1.7	8.3 \pm 0.4	10.0 \pm 1.0	'Inositide P'
'Inositide P'	(IV - V) - (XI - VII)	20.3	9.0	9.6	'Inositide P'

frequently used estimates of the concentration of PNA obtained by combining the procedures of Schmidt & Thannhauser (1945) and Schneider (1945). For white matter and peripheral nerve the figures are greater than those obtained with the ultraviolet absorption method, but for grey matter the differences are not statistically significant ($P = 0.2$ and 0.3 , respectively).

Table 7 shows that the values found for the concentration of 'phosphoprotein' of Schneider (1945), i.e. the phosphorus remaining after the TCA

If it is assumed that the ultraviolet-absorption values are the more reliable, it is clear that the method of Schmidt & Thannhauser (1945), while giving a good estimate of the concentration of DNA, gives a gross overestimate of the concentration of PNA, especially for white matter and, to a lesser extent, for peripheral nerve. Both the colour reactions and the phosphorus estimations of Schneider (1945) also give results that are too high. A hint as to the cause of some of these discrepancies is that the values for the concentration of 'phos-

phoprotein' of Schneider (1945) are much greater than those obtained by the method of Schmidt & Thannhauser (1945), especially for white matter (Table 7). It is clear that much of the phosphorus estimated as 'phosphoprotein' by the former method is measured as nucleic acid, probably as PNA, by the latter. It was decided, therefore, to investigate the extraction of nucleic acid by the Schneider procedure.

Extraction of phosphorus by 5% (w/v) TCA at 90°

The acid-soluble P and lipid P were removed from four pairs of duplicate samples of cat sciatic nerve and the residue was treated for periods of 5, 10, 15 or 30 min., with 5% (w/v) TCA at 90° as described by Schneider (1945). Fig. 1 shows that after 15 min.

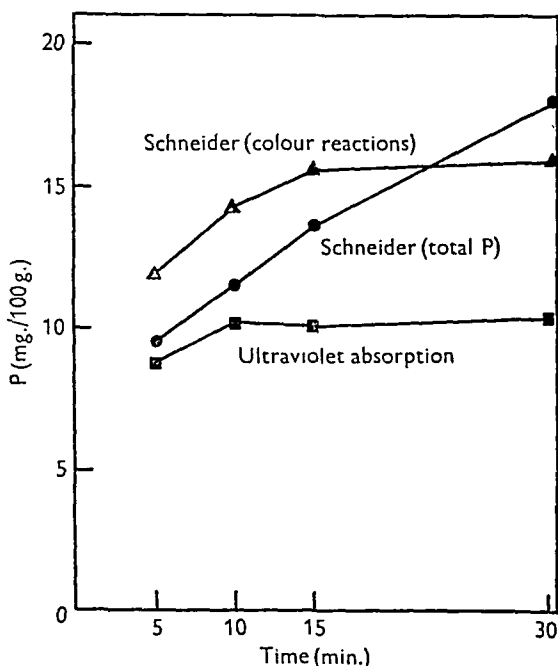


Fig. 1. Effect of time of heating with 5% (w/v) TCA at 90°, on the values obtained for the concentration of total nucleic acid in cat sciatic nerve by three different methods. ▲—▲—▲, colour reactions of Schneider (1945); ●—●—●, total P of extract (Schneider, 1945); ■—■—■, ultraviolet absorption method.

there was little increase in the concentration of nucleic acid in the extract, as measured by the colour reactions (VIII+IX) or ultraviolet absorption (XI), but that the total P in the extract (X) increased with time. At 15 min. (the usual time of extraction in the Schneider procedure) the values were relatively the same as those presented in Table 3. The figure for the colour reactions is the greatest, followed by that for the total P and the ultraviolet absorption, in that order. With an increase in the length of the extraction time, more

phosphorus was removed without any corresponding increase in the ultraviolet absorption, or the value given by the colour reactions. It is clear that some phosphorus compound not possessing the characteristic ultraviolet-absorption properties of nucleic acid or nucleotides was extracted by the TCA. There would thus appear to be no justification for the common practice of designating all phosphorus removed by extraction with TCA for 15 min. as nucleic acid P, and the phosphorus remaining in the residue after this time as 'phosphoprotein' P.

Fig. 2 gives the result of a similar experiment in which both the total P (X) and the ortho-P were measured in the extract and the total P (XII) was measured in the residue. Only a small fraction of the phosphorus of the extract was orthophosphate. In other experiments, in which the time of the extraction was prolonged (2 hr. or more), the residue P tended to zero and the ortho-P in the extract tended to the value found for the Schmidt-Thannhauser 'phosphoprotein' (V). After the usual extraction of 15 min. there was negligible ortho-P in the TCA extract.

Essentially similar results were obtained in a number of other experiments with cat sciatic nerve and with both white matter and grey matter of dog

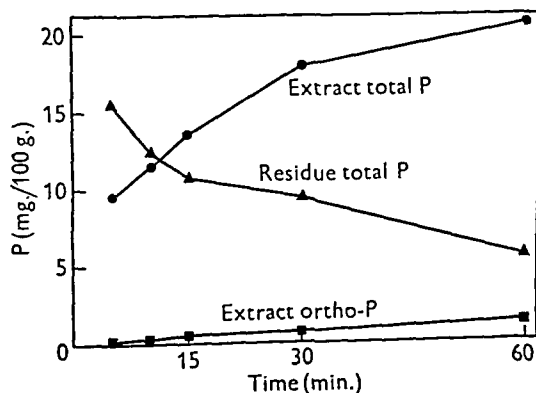


Fig. 2. Effect of time of heating with 5% (w/v) TCA at 90° on the total P and ortho-P of the extract, and the total P of the residue of cat sciatic nerve from which the acid-soluble P and lipid P had previously been removed. ●—●—●, total P of extract; ■—■—■, ortho-P of extract; ▲—▲—▲, total P of residue.

brain. With white matter more phosphorus was extracted by the TCA and the time necessary for complete extraction was longer. At 15 min. the value for the concentration of nucleic acid calculated from total P was often of the same order as that calculated from the colour reactions. With grey matter, on the other hand, the values given by the colour reactions were greater than those calculated from total P. In no instance were we able to confirm the report of Samuels *et al.* (1951) that the concentration of nucleic acid determined by ultra-

violet absorption agreed with the phosphorus values. With other tissues (e.g. pancreas and spleen) the agreement was good.

When the residue remaining after treatment with 5% (w/v) TCA at 90° for 15 min. was subjected to the Schmidt-Thannhauser hydrolysis almost all the material passed into solution and only a further small quantity, containing negligible phosphorus, was precipitated when the solution was neutralized and TCA added to make the final concentration 5%. Table 8 shows that very little of the phosphorus of the residue appeared in the hydrolysate as ortho-phosphate. The concentration of ortho-P was found to be similar to that obtained for the 'phosphoprotein' of Schmidt & Thannhauser (1945) (V) in a parallel experiment.

Table 8. *Residue phosphorus of cat brain after extraction with 5% (w/v) TCA for 15 min. at 90°*

(mg. P/100 g. wet tissue.)

	Experiment	
	1	2
Total residue P (XII)	8.2	11.3
After Schmidt & Thannhauser (1945)		
hydrolysis:		
Ortho-P	3.2	2.1
Organic P	5.0	9.2
'Phosphoprotein' of Schmidt & Thannhauser (1945) (V)	3.5	2.3

Similar concentrations of ortho-P are thus obtained when tissue from the nervous system, from which the acid-soluble and lipid P has been removed, is (a) hydrolysed by the method of Schmidt & Thannhauser (1945), (b) subjected to prolonged extraction (longer than 2 hr.) with 5% (w/v) TCA at 90°, and (c) treated with 5% (w/v) TCA at 90° for 15 min. and the residue subjected to the Schmidt-Thannhauser hydrolysis. The origin of this phosphorus is obscure. Presumably it is derived from an acid-insoluble compound which, like casein, yields ortho-P on hydrolysis. This phosphorus represents the 'phosphoprotein' of Schmidt & Thannhauser (1945) and will be referred to as PP.

The phosphorus, some of which remains in the residue after extraction for 15 min. with 5% (w/v) TCA at 90°, and which is slowly removed by further extraction, is undoubtedly determined as PNA in the Schmidt-Thannhauser procedure and presumably causes the high values when PNA is determined by this method (Table 6). The substance responsible for these high PNA values interferes in the same way as the protein-bound metaphosphate of yeast (Wiame, 1947, 1949; Schimdt, Hecht & Thannhauser, 1946). Protein-bound metaphosphate was not found in animal tissues by Ebel (1949) or Davidson, Frazer & Hutchison (1951).

However, a phosphorus compound with the requisite properties is the trypsin-resistant lipid-protein complex isolated from the white matter of calf brain by Folch & Le Baron (1951). This substance, which contains inositol diphosphate, is not removed from brain tissue by the usual ethanol-ether extraction, and Folch & Le Baron (1951) state that all of the phosphorus of this compound appears as PNA in the method of Schmidt & Thannhauser (1945). Folch (1951) states that this substance occurs chiefly in white matter of brain, and he believes it to be part of the classical 'neurokeratin' described by Kühne & Chittenden (1889) and thought to be a constituent of the myelin sheath of a mammalian nerve fibre. For further comments on the chemical nature of 'neurokeratin', see the paper by Folch & Lees (1951). The phosphorus of the protein-bound P (III) that cannot be accounted for either as nucleic acid or as PP will be referred to as 'inositide P'. It should be stressed that there is no evidence that all such phosphorus is present in brain as inositide, although it is shown (see below) that this type of phosphorus compound occurs in much greater concentration in white matter than in grey matter.

An estimate of the concentration of the 'inositide P' remaining after the removal of acid-soluble P and lipid P can be obtained by subtracting the sum of the values found for the PP (V) and the total nucleic acid determined by ultraviolet absorption (XI) from the protein-bound P (III). By subtracting the ultraviolet-absorption value for PNA (XI - VII) from the value obtained by the Schmidt-Thannhauser method (IV - V), another estimate of 'inositide P' can be obtained by a slightly different method.

Table 7 gives the concentration of 'inositide P' determined by each of these methods. By far the greatest concentration is in the white matter of dog brain, where 'inositide P' accounts for two-thirds of the protein-bound P. The concentration in grey matter and cat sciatic nerve is considerably lower.

*Extraction of chromogenic material by
5% (w/v) TCA at 90°*

Fig. 3 shows the results of an experiment similar to that represented in Fig. 1 in which the colour reactions of Dische (1930) and Mejsbaum (1939) were carried out on TCA extracts of cat sciatic nerve. The extraction times were from 5 to 60 min. Although it is shown in Fig. 1 that the total nucleic acid determined by the colour reactions did not increase when the extraction time was increased from 15 to 30 min., Fig. 3 shows that after 15 min. the colour given by the diphenylamine reaction became less, and that given by the orcinol reaction became greater. These were consistent findings and were repeated in a number of similar experiments

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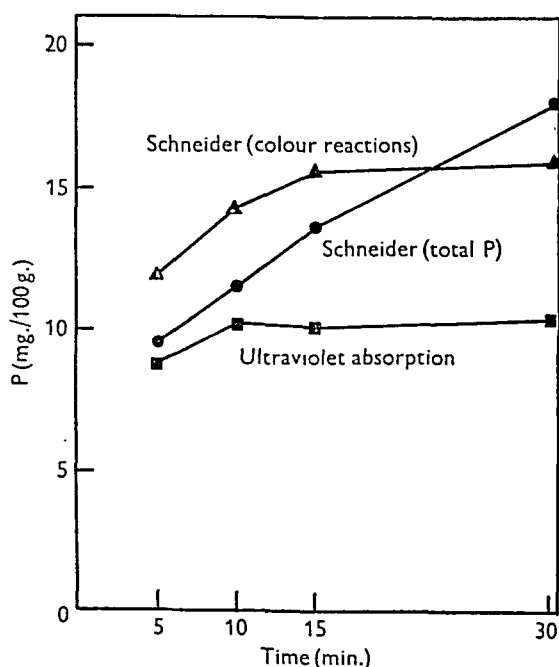


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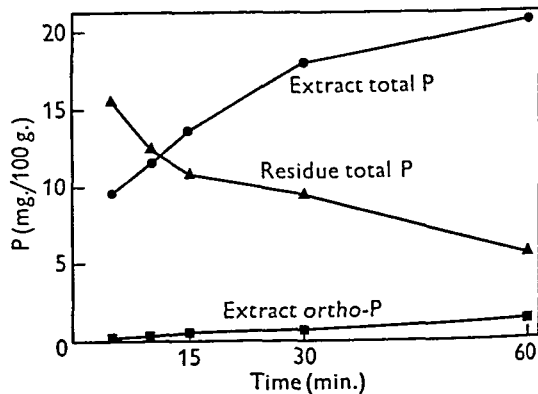


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Table 7 gives the concentration of 'inositide P' determined by each of these methods. By far the greatest concentration is in the white matter of dog brain, where 'inositide P' accounts for two-thirds of the protein-bound P. The concentration in grey matter and cat sciatic nerve is considerably lower.

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with white matter and grey matter of dog brain as well as with cat sciatic nerve.

The lower values for the diphenylamine reaction with extraction times greater than 15 min. were obtained with tissues other than nerve or brain, although the higher values given by the orcinol reaction were peculiar to tissue from the nervous

values obtained were similar to those given by total P (VI) or ultraviolet absorption (VII), and they were much lower than those obtained when the reaction was done on the Schneider TCA extract (VIII). The absorption characteristics of the colour complex given by the isolated DNA appeared normal. In the orcinol reaction the green colour obtained with brain extracts appeared the same as that given by the PNA standard, and the characteristics of the absorption curve of the colour complex were similar.

The results indicate that these two colour reactions are not suitable for estimating nucleic acid in TCA extracts of tissue from the nervous system.

DISCUSSION

It would thus appear that tissue from the nervous system contains at least two substances that interfere greatly with methods widely used for the determination of nucleic acids: (1) the 'inositide P' that is estimated as PNA by the method of Schmidt & Thannhauser (1945) and is measured, in part, as nucleic acid P by the method of Schneider (1945), and (2) chromogenic material that interferes with both the diphenylamine reaction of Dische (1930) and the orcinol reaction of Mejbaum (1939). Table 9 summarizes the substances that would be measured in each of the determinations outlined in 'Analytical methods'. In addition, some of the substances that would be measured in each of the estimates of total nucleic acid, DNA and PNA and 'phosphoprotein' given in Tables 4-7, can be found in the last column of the tables.

No attempt was made to determine whether a phosphorus compound similar to that which gives rise to the 'inositide P' occurs in tissues other than those of the nervous system. The experiments of Davidson *et al.* (1951) would suggest the possibility

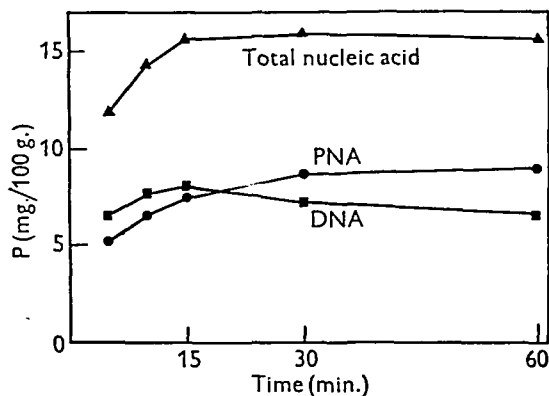


Fig. 3. Effect of time of heating with 5% (w/v) TCA on the values obtained for the concentration of total nucleic acid, DNA and PNA in cat sciatic nerve by the colour reactions. ▲—▲—▲, total nucleic acid by addition; ■—■—■, DNA by method of Dische (1930); ●—●—●, PNA by the method of Mejbaum (1939).

system. With extracts of brain or nerve the colour produced in the diphenylamine reaction was a blue-green rather than the clear blue of the DNA standard. With white matter the peak of the absorption curve of the colour complex was the same as that of the standard, but the curve was much flatter. With grey matter the curve was greatly distorted and the peak was shifted to lower wavelengths. When the reaction was carried out on DNA isolated by the Schmidt-Thannhauser method, the

Table 9. Substances estimated in each of the determinations outlined in 'Analytical methods'

Determination	Previously assumed	Estimated
I	Acid-soluble P	Acid-soluble P
II	Lipid P	Lipid P
III	DNA + PNA + 'phosphoprotein' of Schmidt & Thannhauser (1945)	DNA + PNA + PP + 'inositide P'
IV	PNA + 'phosphoprotein' of Schmidt & Thannhauser (1945)	PNA + PP + 'inositide P'
V	'Phosphoprotein' of Schmidt & Thannhauser (1945)	PP
VI	DNA of Schmidt & Thannhauser (1945)	DNA
VII	DNA by ultraviolet absorption	DNA
VIII	DNA by Dische (1930) diphenylamine reactions (Schneider, 1945)	DNA + ?
IX	PNA by Mejbaum (1939) orcinol reaction (Schneider, 1945)	PNA + ?
X	DNA + PNA by phosphorus (Schneider, 1945)	DNA + PNA + part of 'inositide'
XI	DNA + PNA by ultraviolet absorption	DNA + PNA
XII	'Phosphoprotein' of Schneider (1945)	PP + part of 'inositide P'

that smaller concentrations of such a compound may be present in liver. In most tissues the concentration of nucleic acid is considerably greater than in brain or nerve, and the Schneider 'phosphoprotein' (XII) represents a much smaller percentage of the total protein-bound P. The effect on the quantitative determination of nucleic acid would, therefore, be much less. However, Jeener (1949) and Davidson *et al.* (1951) have shown that after the administration of ^{32}P the specific activity of the Schmidt-Thannhauser PNA fraction of liver (our IV-V) is much greater than that of isolated PNA, even after the very active PP (our V) has been removed. These results suggest that liver contains small amounts of a metabolically active phosphorus compound that interfere with specific activity measurements made by the Schmidt-Thannhauser method. This interference is much greater in tissue of the nervous system. 'Inositide P' accounts for over 80% of the Schmidt-Thannhauser PNA (IV-V) in the white matter of dog brain (Table 5) and Strickland (1951), working in this laboratory, has shown that ^{32}P is incorporated into the PP and 'inositide P' of brain and nerve much more readily than into the DNA or PNA.

The gross differences between the estimates of the concentration of nucleic acids obtained by the methods of Schmidt & Thannhauser (1945), Schneider (1945) and by ultraviolet absorption are greatest in tissue of the nervous system. In a number of experiments with pancreas, spleen, thymus, white cells and reticulocytes, in which the same techniques and the same standards were used, fair agreement was obtained between the methods. This would strongly suggest that the differences observed for the nervous system are genuine. Since each of the three methods is dependent upon a different part of the nucleic acid molecule, it is at least theoretically possible that the lack of agreement is due to some fundamental difference in the structure of the nucleic acids of the nervous system. We are aware of no studies on the chemistry of nucleic acids isolated from the nervous system, but in view of recent studies on nucleic acid structure (i.e. Chargaff, 1951) such a possibility would appear to be unlikely.

Both Schneider (1946) and Tsuboi (1950) reported good agreement between various nucleic acid methods for a number of tissues, although it is apparent from the paper of Schneider (1946) that in rat brain the value for the concentration of total nucleic acid, determined by the Schmidt-Thannhauser method, is greater than that determined either by total P or by colour reactions done on the Schneider extract. The difference is due to an increase in the figure for PNA. Schneider (1945, 1946) also reported that for brain tissue the concentration of total nucleic acid in the TCA extract given by the

colour reactions agreed with the figure given by phosphorus estimations. This agreement is fortuitous. The phosphorus figures are too high because of the presence of some 'inositide P', and the figures given by the colour reactions are too high because of interfering chromogenic material.

The results obtained by the ultraviolet absorption method for the concentration of nucleic acid in brain and nerve are much lower than the figures published by previous workers (Kossel, 1882; Berenblum, Chain & Heatley, 1939; Rosenthal & Drabkin, 1943; Davidson & Waymouth, 1944; Schmidt & Thannhauser, 1945; Schneider, 1945, 1946; Schneider & Klug, 1946; von Euler & Hahn, 1948; Bodian & Dziewiatkowski, 1950; Samuels *et al.* 1951). In Table 10 our results are compared with those obtained in the recent studies of Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951). As already pointed out, our results for acid-soluble and lipid P in the white matter and grey matter of dog brain and cat sciatic nerve agree well with the published figures for monkey brain and guinea-pig nerve, but our figures for total nucleic acid, DNA, and 'phosphoprotein' are considerably lower.

It is of interest to note that the concentration of total nucleic acid in grey matter is considerably greater than that in white matter. White matter and grey matter of dog brain and cat sciatic nerve all contain similar concentrations of DNA, but there is a much higher concentration of PNA in grey matter. This is not surprising, since grey matter contains many more nerve cell bodies. These contain abundant Nissl material, which is believed to be rich in PNA. White matter and grey matter of dog brain and cat sciatic nerve also contain a similar concentration of PP (V).

The finding of Bodian & Dziewiatkowski (1950) that white matter contains more 'phosphoprotein' (our XII) than grey matter is confirmed, although the values for dog brain reported in Table 7 are somewhat lower, both for white matter and for grey matter, than the figures given by these workers for monkey brain. The high concentration of phosphorus in the 'phosphoprotein' fraction of white matter is due to the much greater concentration of 'inositide P', much of which remains in this fraction after the TCA extraction. The high concentration of 'inositide P' in white matter, taken in conjunction with our further finding that this substance disappears from a peripheral nerve during Wallerian degeneration, even although the concentration of nucleic acid increases (Logan, Mannell & Rossiter, 1952), gives added weight to the suggestion of Folch (1951) that the 'inositide P' is derived from the 'neurokeratin' of the myelin sheath. It is this substance and not nucleic acid that contributes to the high protein-bound P found in white matter by Davidson & Waymouth (1944).

SUMMARY

1. The concentration of acid-soluble phosphorus, lipid phosphorus and protein-bound phosphorus was determined in the white matter and the grey matter of dog brain and in the sciatic nerve of the cat. In addition, the concentration of total nucleic acid, deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA) and 'phosphoprotein' was determined by the methods of Schmidt & Thannhauser (1945), Schneider (1945), and by an ultraviolet absorption method.

2. The figures for total nucleic acid, DNA and PNA given by the method of Schneider (1945), and those for total nucleic acid and PNA given by the method of Schmidt & Thannhauser (1945), were greater than the corresponding figures given by the ultraviolet absorption method. With other tissues there was good agreement between the three methods.

3. An investigation of the causes of these discrepancies revealed that tissue of the nervous system contains (a) a phosphorus compound, probably the inositol-containing trypsin-resistant lipid-protein complex of Folch & Le Baron (1951), which is estimated as PNA by the method of Schmidt &

Thannhauser (1945) and is measured, in part, as nucleic acid phosphorus in the method of Schneider (1945), and (b) chromogenic material that interferes with the colour reactions in the method of Schneider (1945).

4. The values obtained for the concentration of nucleic acid in tissue from the nervous system by the method of ultraviolet absorption are considerably lower than those previously published in the literature. The concentration of DNA is of the same order in white matter and grey matter of dog brain and in cat sciatic nerve, but the concentration of PNA is much greater in the grey matter.

5. The concentration of 'phosphoprotein' (PP) is of the same order in white matter and grey matter of dog brain and in cat sciatic nerve, but the concentration of 'inositide P' is much greater in white matter.

6. Some comments on the determination of DNA and PNA in tissue from the nervous system by the method of ultraviolet absorption are given in the Appendix.

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APPENDIX

A Note on the Determination of Deoxypentosenucleic Acid and Pentosenucleic Acid in Tissue from the Nervous System by Ultraviolet Absorption

By J. E. LOGAN, W. A. MANNELL AND R. J. ROSSITER

Department of Biochemistry, University of Western Ontario, London, Canada

Schneider (1945) introduced the convenient method of extracting nucleic acids from tissue with 5% (w/v) TCA. Such an extract, which contains minimal quantities of protein, may be used for the determination of nucleic acid by ultraviolet absorption.

The tissue samples were prepared as described in 'Analytical methods'. The extinction of (a) the TCA extract containing the DNA (VII) and (b) the TCA extract containing the total nucleic acid (XI) was determined in a Beckman Model DU quartz spectrophotometer at 268.5 m μ . The concentration of nucleic acid (in mg. P/100 g. wet tissue) for both DNA and total nucleic acid was calculated on the assumption that both DNA and PNA have an ϵ (P) of 9850 at this wavelength (see below). The concentration of PNA was determined by difference.

The procedure presented certain difficulties worth recording.

(1) Change in absorption characteristics of standards on heating for 15 min. at 90° in 5% (w/v) TCA

After heating in 5% (w/v) TCA at 90° for 15 min. the absorption maximum of the standard DNA was shifted from 259 to 267 m μ . and the ϵ (P) at maximum absorption increased from 7300 to 9900 (Fig. 4). After neutralizing and buffering in 0.1 M-phosphate at pH 7 the absorption maximum returned to 263 m μ ., but the extinction remained high. The changes in the standard PNA were in the same direction, but much less marked. After neutralizing and buffering at pH 7 the absorption maximum returned to

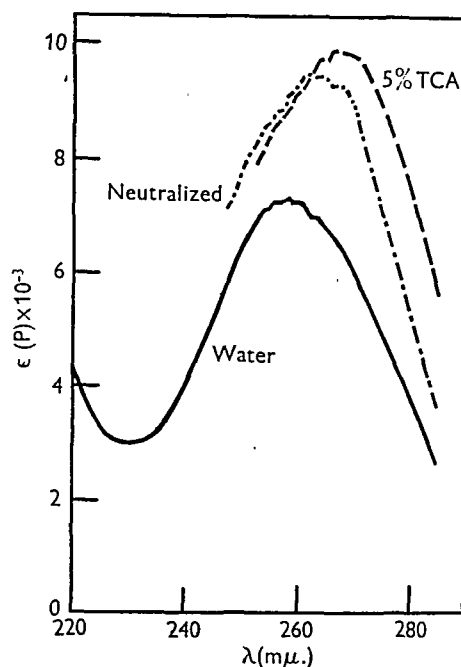


Fig. 4. Ultraviolet absorption of standard DNA. —, in water; — — —, after heating with 5% (w/v) TCA at 90° for 15 min.; — · — ·, after heating with TCA as above, neutralizing and buffering in 0.1 M-phosphate at pH 7.

Table 11. *Effect of heating standard preparations of DNA and PNA for 15 min. at 90° in 5% (w/v) TCA*

	Standards used		Calculated for data of Tsuboi (1950)	
	$\lambda_{\max.}$ (m μ .)	ϵ (P) at $\lambda_{\max.}$	$\lambda_{\max.}$ (m μ .)	ϵ (P) at $\lambda_{\max.}$
DNA:				
In distilled water	259	7300	259	6900
After heating for 15 min. at 90° in 5% (w/v) TCA	267	9900	—	—
As above, but neutralized and buffered in 0.1 M-phosphate at pH 7.0	263	9500	262	9600*
PNA:				
In distilled water	258	9300	259	8300
After heating for 15 min. at 90° in 5% (w/v) TCA	261	10700	—	—
As above, but neutralized and buffered in 0.1 M-phosphate at pH 7.0	259	11000	259	10000*

* Heated for 20 min. at 90° in 5% (w/v) TCA. Readings at 260 m μ .

259 m μ . (Table 11). Similar changes in the ϵ (P) and λ_{\max} of the standard DNA and PNA solutions were observed after heating in 6% (w/v) HClO₄ at 90° for 15 min. Other workers have described changes in the ultraviolet absorption characteristics of nucleic acids after acid, alkaline, or enzymic hydrolysis (Warren & Chanutin, 1948; Tsuboi, 1950; Little & Butler, 1951).

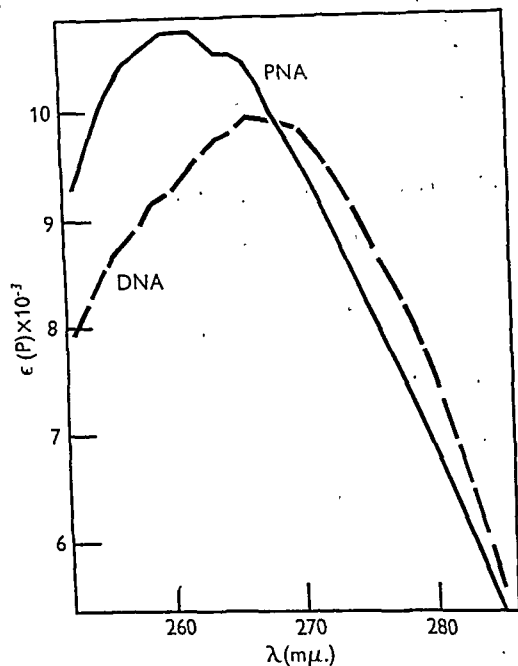


Fig. 5. Ultraviolet absorption of standard DNA and standard PNA after heating with 5% (w/v) TCA at 90° for 15 min. — — —, DNA; —, PNA.

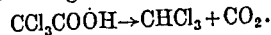
Tsuboi (1950) reported similar changes in the λ_{\max} and ϵ of DNA and PNA solutions. Table 11 shows that the wavelength of maximum absorption, and the ϵ (P) of both the standard DNA and the standard PNA after treatment with TCA and neutralization, agree with results obtained by Tsuboi (1950).

The absorption curves of equal concentrations (on a P basis) of DNA and PNA were found to intersect at 268.5 m μ . when the measurements were made after heating in TCA without neutralization (Fig. 5). Accordingly, routine measurements were made at this wavelength where both the DNA and PNA have an ϵ (P) of 9850. This wavelength also has the added advantage that the absorption due to the TCA is much less than at 260 m μ . (see below).

(2) Ultraviolet absorption of TCA

Fig. 6 shows the great ultraviolet absorption of 5% (w/v) TCA. On the same graph is plotted the extinction of PNA (2 μ g. P/ml.). It can be seen that at 260 m μ . the extinction due to the TCA is of the same order as that due to the nucleic acid. As a result, all reference blanks and standards must contain exactly the same concentration of TCA as does the test solution. In addition, the method suffers from the disadvantage that heating at 90° for 15 min. causes con-

siderable destruction of TCA (Table 12), the TCA being broken down according to the equation



Because of this, the blanks and standards must be heated for exactly the same length of time as the test samples. Various attempts were made to obviate this difficulty. The TCA could be removed with solvents such as ether or ethyl acetate, but this was time-consuming and in samples of

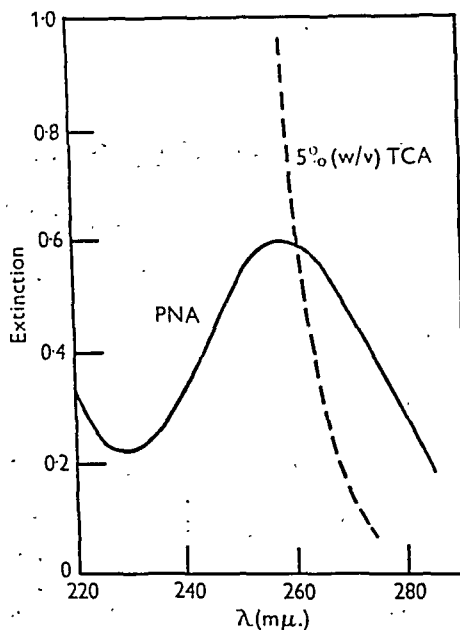


Fig. 6. Ultraviolet absorption of TCA and standard PNA. — — —, 5% (w/v) TCA; —, PNA (2 μ g. P/ml.).

biological tissue emulsions proved troublesome. HClO₄ (6%, w/v), which is free from interfering ultraviolet absorption, was found to extract the nucleic acids. However, when HClO₄ was used with tissue from the nervous system, the extracts absorbed strongly in the region of 274–276 m μ ., presumably due to protein or protein degradation products. Experiments with casein showed that, when the 5% (w/v) TCA was replaced by 6% (w/v) HClO₄, more than three times the amount of material absorbing at 274–276 m μ . was extracted.

Table 12. Extinction at 269 m μ . of 5% (w/v) TCA heated at 90°

Heating time (min.)	$E_{1\text{cm.}}^{5\%}$ at 269 m μ .	
	Exp. 1	Exp. 2
0	0.162	0.162
5	0.158	0.157
10	0.150	0.150
15	0.135	0.132
30	0.113	0.114
60	0.092	0.090

As a routine, therefore, no attempt was made to remove the TCA. At 268.5 m μ . the proportionate effect due to any slight difference between the concentration of TCA in the test solution and in the reference blank is much less than at 260 m μ . (Fig. 6).

(3) *Extraction of nucleic acids*

Fig. 1 shows that the absorption at 268.5 m μ . of a TCA extract of cat sciatic nerve was not significantly greater when the extraction time was increased from 15 to 30 min. Similar results were obtained with white matter and grey matter of dog brain. However, after prolonged heating with TCA (2 hr.) a slight increase in the absorption at 268.5 m μ . was observed. When the residue remaining after a 15 min.

extraction with TCA was extracted for a further 105 min., the extract had a slight absorption at 268 m μ ., but the maximum absorption was at 274–275 m μ ., presumably due to protein degradation products.

In the standard 15 min. extract of brain or nerve there was no suggestion of a secondary peak at 274–276 m μ . as was observed with 6% (w/v) HClO₄. It is felt that interference due to protein degradation products in samples heated in 5% (w/v) TCA for no longer than 15 min. is minimal.

Chemical Studies of Peripheral Nerve During Wallerian Degeneration

3. NUCLEIC ACIDS AND OTHER PROTEIN-BOUND PHOSPHORUS COMPOUNDS

By J. E. LOGAN, W. A. MANNELL AND R. J. ROSSITER

Department of Biochemistry, University of Western Ontario, London, Canada

(Received 19 November 1951)

This paper describes changes in the concentration of nucleic acids and other protein-bound phosphorus compounds in the sciatic nerve of the cat after nerve section or nerve crush. If a nerve is cut (neurotmesis) that portion of the nerve peripheral to the point of section undergoes the familiar series of changes known as Wallerian degeneration. If the nerve is crushed, a procedure that interrupts the axons and myelin sheaths but leaves the connective tissue sheaths intact (axonotmesis), the nerve degenerates as before. After a short latent period the degenerating peripheral segment is re-innervated by axon tips from the intact central stump. There is thus a regeneration superimposed upon the degeneration. In previous publications from this laboratory changes in the concentration of lipids after nerve section or nerve crush have been described (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950).

While this work was in progress, papers by Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) appeared giving some figures for protein-bound phosphorus compounds in peripheral nerve during Wallerian degeneration. By using the method of ultraviolet absorption described in the preceding paper (Logan, Mannell & Rossiter, 1952), a more reliable estimate of the concentration of total nucleic acid in a degenerating peripheral nerve has been obtained. In addition, the changes that occur in the concentration of deoxypentose-nucleic acid (DNA), pentosenucleic acid (PNA), 'phosphoprotein' (PP) and 'inositide P' after nerve section or nerve crush have been described for the first time. The figures given for 'inositide P' probably represent the phosphorus of the inositol-containing trypsin-resistant lipid-protein complex

described by Folch & Le Baron (1951) and thought by Dr Jordi Folch to be part of the 'neurokeratin' of Kühne & Chittenden (1889).

METHODS

The right sciatic nerves of fifty-two cats were either sectioned or crushed at the level of the greater trochanter of the femur. Details of the operations have already been described (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex or weight of the animals. After intervals of time varying from 2 to 600 days the animals were killed, and the segment of nerve distal to the site of the operation was removed. At the same time, a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue, weighed, and the phosphorus compounds were estimated as described in the preceding paper (Logan *et al.* 1952). The nucleic acids were estimated by ultraviolet absorption.

Because of the great increase in the wet weight of degenerating and regenerating nerves, the results have all been expressed in terms of the fresh weight of a similar length of the intact nerve of the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been sectioned or crushed, i.e. at zero time. All analyses were done in duplicate.

RESULTS

Nerve section

Table 1 shows that 16 days after nerve section there was an increase in the concentration of acid-soluble P and protein-bound P, i.e. phosphorus remaining after the removal of acid-soluble P and lipid P, and a decrease in the concentration of lipid P and total P. Table 2 shows that the increase in the concentration of protein-bound P was due to an increase (by a

factor of 3 to 4) in the concentration of nucleic acid. The concentration of both DNA and PNA increased, but the increase in the concentration of PNA was greater than that of DNA, causing a rise in the PNA/DNA ratio. Neither PP nor 'inositide P' contributed to the rise in the protein-bound P. After 16 days the mean concentration of PP was less than that in the control nerves. The mean concentration of 'inositide P' was also less than that in the control nerves, although the difference is not

Figs. 1-5 show the changes in the concentration of the various phosphorus compounds at different times after nerve section. The concentration of acid-soluble P (Fig. 1) increased, reaching a maximum at 8-16 days and then decreased to values well below those of the control nerves. As was found in the previous study (Johnson *et al.* 1949), the concentration of lipid (Fig. 2) did not change greatly during the first 8 days and then decreased rapidly during the period 8-32 days.

Table 1. *Phosphorus compounds in sciatic nerve of the cat 16 days after nerve section or nerve crush*

(Results are expressed as mg. P/100 g. wet tissue. No. of animals is stated in parentheses under each result. *P* gives the significance of the difference between values for experimental and control nerves.)

Estimation	Control		16 days after nerve section			16 days after nerve crush		
	Mean	S.E.M.	Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>
Acid-soluble P	52.2 ± 0.7 (42)		62.1 ± 2.1 (6)		<0.01	64.9 ± 2.5 (5)		<0.01
Lipid P	310 ± 5.6 (50)		156 ± 20 (6)		<0.01	149 ± 14 (5)		<0.01
Protein-bound P	19.4 ± 0.5 (52)		41.7 ± 1.2 (6)		<0.01	41.5 ± 2.2 (5)		<0.01
Total P (by addition)	380.8 ± 5.8 (42)		259.7 ± 33.8 (6)		<0.01	254.9 ± 11.7 (5)		<0.01

Table 2. *Protein-bound P in sciatic nerve of the cat 16 days after nerve section or nerve crush*

(Results are expressed as mg. P/100 g. wet tissue. No. of animals is stated in parentheses under each result. *P* gives the significance of the difference between values for experimental and control nerves.)

Estimation	Control		16 days after nerve section			16 days after nerve crush		
	Mean	S.E.M.	Mean	S.E.M.	<i>P</i>	Mean	S.E.M.	<i>P</i>
Total protein-bound P	19.4 ± 0.5 (52)		41.7 ± 1.2 (6)		<0.01	41.5 ± 2.2 (5)		<0.01
Total nucleic acid	8.7 ± 0.2 (52)		32.9 ± 1.1 (6)		<0.01	34.5 ± 2.3 (5)		<0.01
DNA	4.8 ± 0.1 (52)		14.4 ± 0.9 (6)		<0.01	13.4 ± 0.5 (5)		<0.01
PNA	3.9 ± 0.1 (52)		18.5 ± 0.8 (6)		<0.01	21.1 ± 2.4 (5)		<0.01
PP	1.8 ± 0.1 (51)		1.0 ± 0.1 (6)		<0.01	1.2 ± 0.1 (5)		<0.01
'Inositide P'	9.0 ± 0.4 (51)		7.7 ± 0.8 (6)		<0.3	5.8 ± 1.2 (5)		<0.02
PNA/DNA ratio	0.9 (52)		1.3 (6)			1.6 (5)		

significant statistically ($P=0.2-0.3$). This is no doubt due to the lack of precision in the 'inositide P' determination. 'Inositide P' is obtained by difference from three individual determinations. After 32 days, however, the mean concentration of 'inositide P' in the degenerating nerves was very much less than that in the control nerves and the difference is statistically significant. The standard errors given in Tables 1 and 2 indicate the degree of reproducibility to be expected with different animals.

Fig. 3 shows that the concentration of protein-bound P rose to a maximum at 16 days and then fell slowly throughout the course of the degeneration. The rise, which was apparent after only 2 days of degeneration, was due to an increase in the concentration of nucleic acid. There was a steady fall in the concentration of PP. The concentration of 'inositide P', like the concentration of lipid P, did not change greatly in the first 8 days and then fell sharply in the period 8-32 days.

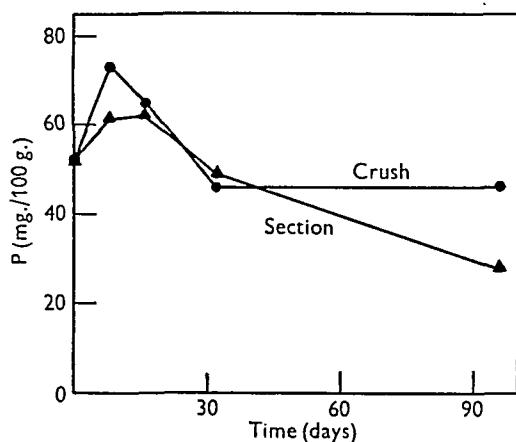


Fig. 1. Acid-soluble P of cat sciatic nerve after nerve section (▲—▲) or nerve crush (●—●). Each point represents the mean for three or more animals.

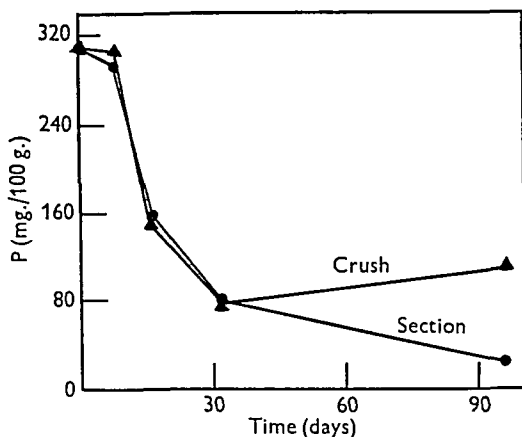


Fig. 2. Lipid P of cat sciatic nerve after nerve section (●—●) or nerve crush (▲—▲). Each point represents the mean for three or more animals.

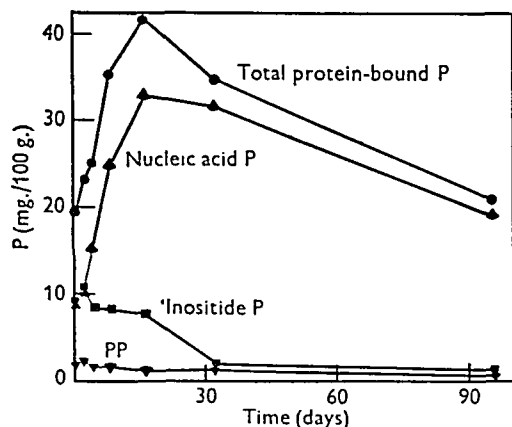


Fig. 3. Protein-bound P of cat sciatic nerve after nerve section. ●—●, total protein-bound P; ▲—▲, nucleic acid P; ■—■, 'inositolide P'; ▼—▼, PP. Each point represents the mean for three or more animals.

Fig. 4 gives the concentrations of DNA and PNA. Both nucleic acids increased in concentration, but PNA increased more rapidly than DNA, so that by 32 days the PNA/DNA ratio had increased from 0.9 to 2.0 (Fig. 5). After 32 days the PNA/DNA ratio fell gradually.

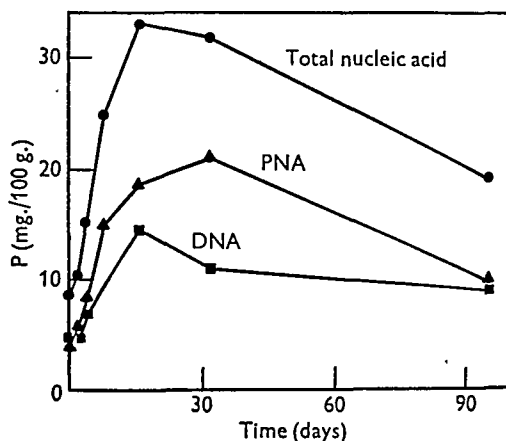


Fig. 4. Nucleic acids of cat sciatic nerve after nerve section. ●—●, total nucleic acid; ■—■, DNA; ▲—▲, PNA. Each point represents the mean for three or more animals.

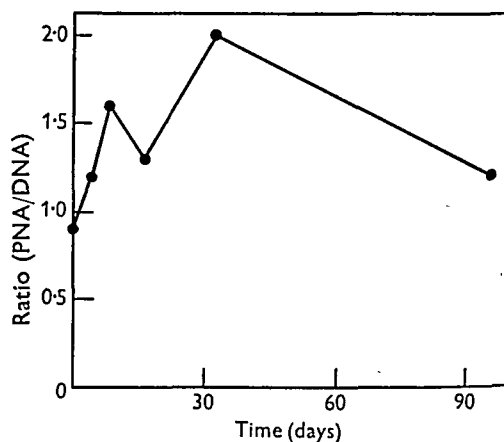


Fig. 5. Ratio of PNA to DNA in cat sciatic nerve after nerve section. Each point represents the mean for three or more animals.

Nerve crush

Tables 1 and 2 show that 16 days after nerve crush the phosphorus compounds changed in the same direction and to the same extent as after nerve section. None of the figures for the crushed nerves is significantly different from that for the corresponding substance in the sectioned nerves.

By 96 days signs of regeneration began to appear in the crushed nerves. The concentration of acid-soluble P (Fig. 1) and lipid P (Fig. 2) was greater than that in the sectioned nerves ($P < 0.01$, in both instances). With much longer periods of regenera-

tion the concentration of total P and lipid P continued to increase (Fig. 6), and the concentration of protein-bound P continued to fall. Fig. 7 shows that the fall in protein-bound P was due to a fall in the concentration of nucleic acid. By 96 days the concentration of both PP and 'inositide P' had commenced to increase, even although the concentration

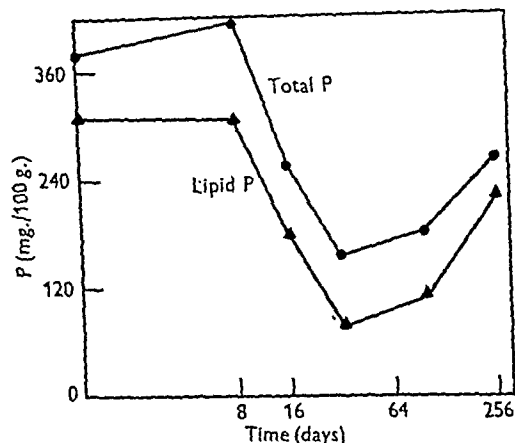


Fig. 6. Total P (●—●) and lipid P (▲—▲) of cat sciatic nerve after nerve crush. Each point represents the mean for three or more animals.

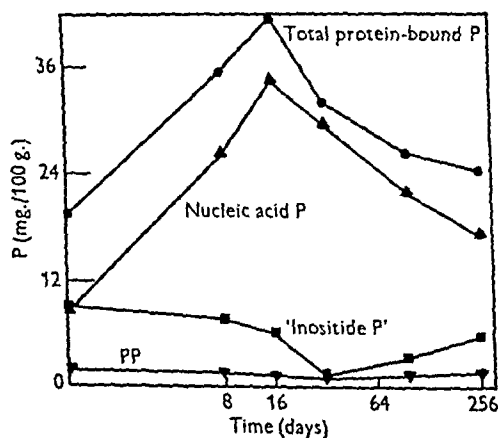


Fig. 7. Protein-bound P of cat sciatic nerve after nerve crush. ●—●, total protein-bound P; ▲—▲, nucleic acid P; ■—■, 'inositide P'; ▼—▼, PP. Each point represents the mean for three or more animals.

of protein-bound P was still falling. Both DNA and PNA contributed to the fall in the concentration of nucleic acid, but the PNA decreased more rapidly than the DNA, so that the PNA/DNA ratio, which in the period 8–32 days was greatly in excess of the figure for the control nerves, fell steadily, reaching a value below that of the controls by 250 days (Fig. 8).

After 250 days, at a time when the acid-soluble P and PP had returned to normal, the concentration of both lipid P and 'inositide P' was still consider-

ably less than that in the control nerves and the concentration of nucleic acid, especially of DNA, was greater. In four additional animals the nerves were crushed and then allowed to regenerate for 550–600 days after the operation. Even after this length of time the concentration of lipid P and 'inositide P' was still less than that in the control nerves of the opposite side and the concentration of nucleic acid was still greater.

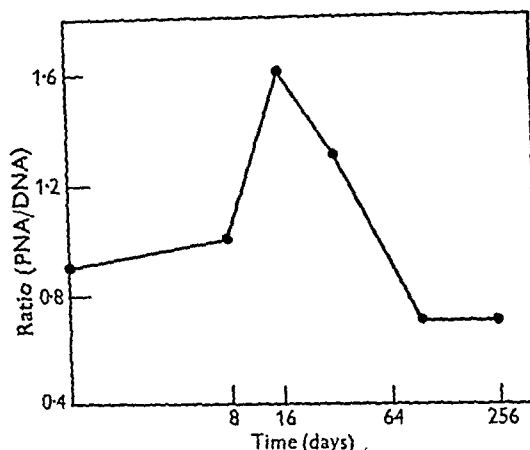


Fig. 8. Ratio of PNA to DNA in cat sciatic nerve after nerve crush. Each point represents the mean of three or more animals.

DISCUSSION

When a peripheral nerve is sectioned or crushed that portion of the nerve distal to the site of the lesion degenerates. The axon disintegrates and the myelin sheath at first fragments and ultimately is destroyed. The Schwann cells proliferate and the endoneurium and neurilemma thicken to form a Schwann tube, which is able to receive a growing axon tip from the intact central stump. The degeneration is, in a sense, a preparation for regeneration.

The sharp fall in the concentration of lipid P during the period 8–32 days after either type of operation is most likely the result of the destruction of the myelin sheath (Johnson, McNabb & Rossiter, 1950). The finding that the 'inositide P' also disappears at this time is of interest. If this fraction represents the lipid-protein complex of Folch & Le Baron (1951) and is part of the classical 'neurokeratin', the observation provides good evidence that 'neurokeratin' is a constituent of the myelin sheath of a peripheral nerve fibre. This view is strengthened by the further finding that during the deposition of myelin in nerves regenerating after a crush, the 'inositide P' returns to the nerve at the same time as the lipid P.

The rise in the concentration of nucleic acid, reaching a peak 16–32 days after nerve section or

nerve crush, is probably accounted for by the increase in the number of Schwann cells and endoneurial cells (chiefly fibrocytes and macrophages) that takes place during this time (Young, 1942; Abercrombie & Johnson, 1946). If, as has been suggested, the mean quantity of DNA per diploid cell is constant for any one species (see Davidson, Leslie & White, 1951, for references), it is apparent that the total amount of DNA in a nerve is an index of the number of cell nuclei present. The curve representing the change in the concentration of DNA with time (Fig. 4) is of the same general form as that reported by Abercrombie & Johnson (1946) for the change in the total number of cell nuclei in the degenerating sciatic nerve of the rabbit. Quantitatively the curves are different, but this is not surprising when the many differences between the two sets of experiments are taken into consideration. Our experiments were with cats, not rabbits, and the chemical determination gives an estimate of the number of cell nuclei throughout the whole length of the nerve rather than the number at one selected level. Also it is now known that the extent of the increase in the total number of cell nuclei in a peripheral nerve during Wallerian degeneration depends upon the size and the degree of myelination of the constituent fibres (Joseph, 1947, 1948; Thomas, 1948).

The concentration of PNA gives an indication of the total mass of cytoplasm within the nerve. During the period of cellular proliferation the PNA/DNA ratio, i.e. the concentration of PNA per cell, is considerably greater than that in the control nerves. At this time an increase in the amount of Schwann cell cytoplasm can be demonstrated histologically (Young, 1942). There is also an increase in the concentration of acid-soluble P.

The increase in the concentration of nucleic acid is statistically significant as early as 2 days after the operation, yet even at 8 days, when the concentration of nucleic acid is greatly in excess of that in the control nerves, the concentration of lipid P and 'inositide P' is not significantly decreased. This would indicate that the cellular proliferation commences well in advance of the chemical degradation of the myelin sheath, although, with the degeneration of the axon in the first few days after nerve section, there is a physical destruction of the myelin sheath, with segmentation and ovoid formation (Young, 1945; Johnson *et al.* 1950).

In the sectioned nerves there was no post-mortem or functional evidence of regeneration 96 days after the operation, and in other animals similarly treated this absence of regeneration was confirmed microscopically. In the crushed nerves, on the other hand, myelination had spread throughout the length of the nerve and function appeared normal. Yet even after 600 days, neither the lipid P

nor the 'inositide P' had returned to normal. At this time the regenerating nerves still contained more nucleic acid than the control nerves of the opposite side. This finding is of interest in view of the slow restitution of fibre pattern (Gutman & Sanders, 1943; Sanders, 1948) and full physiological activity (Berry, Grundfest & Hinsey, 1944; Sanders & Whitteridge, 1946; Erlanger & Schoepfle, 1946) in regenerating nerves.

The figures for the concentration of phosphorus compounds in the control nerves agree well with those reported in the previous paper (Logan *et al.* 1952) for a smaller series of normal cat sciatic nerves.

May (1930), Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) have studied the distribution of phosphorus compounds in peripheral nerves during Wallerian degeneration. Comparison of our results with those of May (1930) is of little value because of the great differences in the analytical techniques employed. Bodian & Dziewiatkowski (1950) reported a fall in the concentration of lipid P and a slight increase (of the order of 18%) in the concentration of protein-bound P 7–23 days after section in monkey nerves. Because the 'inositide P' accounts for such a high percentage of the protein-bound P of the control nerves and the concentration of 'inositide P' falls while that of nucleic acid rises during Wallerian degeneration, the percentage increase in the concentration of nucleic acid would be much greater than that of the protein-bound P. In our experiments the protein-bound P of cat sciatic nerve increased by 116% in 16 days, whereas the concentration of nucleic acid increased by 280%. Samuels *et al.* (1951), using the method of Schneider (1945), also reported an increase in the concentration of nucleic acid (112%) in guinea-pig nerves 16 days after section. For the reasons given by Logan *et al.* (1952), the figures of Samuels *et al.* (1951) for nucleic acid, especially those for the control nerves, are probably too high.

SUMMARY

1. The concentration of acid-soluble phosphorus, lipid phosphorus, protein-bound phosphorus, total nucleic acid, deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA), phosphoprotein (PP) and 'inositide phosphorus' was determined in the sciatic nerve of the cat at intervals of time from 2 to 96 days after nerve section (neurotmesis) and 8–600 days after nerve crush (axonotmesis).

2. The acid-soluble phosphorus increased to a maximum at 8–16 days after either nerve section or nerve crush, returning to values not significantly different from those of the control nerves by 32 days. The acid-soluble phosphorus then remained normal in the crushed nerves, but fell to lower values in the sectioned nerves.

3. The lipid phosphorus changed little during the first 8 days and then decreased rapidly during the period 8-32 days after either nerve section or nerve crush. In the sectioned nerves the lipid phosphorus fell still further, whereas in the crushed nerves it had commenced to increase by 96 days and continued to increase throughout the period of regeneration.

4. The concentration of total nucleic acid increased after either nerve section or nerve crush, reaching a maximum in 16 days. Thereafter the concentration of nucleic acid fell in both the sectioned and crushed nerves. After each type of operation the PNA increased more rapidly than the DNA, so that there was an increase in the PNA/DNA ratio.

5. The PP fell throughout the whole course of the degeneration in the sectioned nerves. In the crushed nerves it had commenced to rise by 96 days, and by

250 days it approximated the concentration in the control nerves.

6. The 'inositide phosphorus' followed a course similar to that of the lipid phosphorus. After either type of operation there was little change during the first 8 days, followed by a rapid fall during the period 8-32 days. In the sectioned nerves the 'inositide phosphorus' fell still further, whereas in the crushed nerves it had commenced to increase by 96 days and continued to rise during the entire period of study.

7. The increase in the concentration of nucleic acid and the decrease in the concentration of lipid phosphorus and 'inositide phosphorus' still persisted 600 days after nerve crush.

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A Study of the Amino-acid Complexes Formed by Metals of Group II of the Periodic Classification

By D. J. PERKINS

Department of Chemical Pathology, St George's Hospital Medical School, London, S.W. 1

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Albert (1950) published a study of the formation of complexes between a number of metals and α -amino-acids containing only two ionizing groups. The mathematical treatment of the results from potentiometric titrations enabled the stabilities of the metal complexes to be calculated. Using his potentiometric method all the metals of Group II of the periodic classification were studied in their relation

to glycine, its derivatives and other α -amino-acids with only two ionizing groups. In this paper an attempt has been made to study the effect on complex formation of (a) the nature of the metal, and (b) substitution in the amino-acid. An attempt was also made to correlate the results obtained by potentiometry with the solubility studies of Monk (1951a, b).

EXPERIMENTAL

Materials. The following salts were used in 0.01M-solution: $\text{BaCl}_2 \cdot 6\text{H}_2\text{O}$; SrCl_2 ; CaCl_2 ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$; $\text{Hg}(\text{NO}_3)_2$; $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$; $\text{Pb}(\text{NO}_3)_2$. $\text{Hg}(\text{NO}_3)_2$ was made up in 0.025M- HNO_3 and excess acid back-titrated in the presence of the amino-acid investigated. AgNO_3 was used in 0.02M-solution. All salts were of A.R. quality.

All the amino-acids were vacuum-dried at 100° for at least 48 hr., weighed and made up in 0.02M-solution. Because of the low solubility of their complexes it was necessary to use 0.001M-concentrations of DL-norvaline and DL-norleucine. The purity of each amino-acid was assessed by two-dimensional chromatography and by potentiometric titration with 0.100M-KOH. All samples except DL- α -alanine gave titration curves which on calculation yielded constant pK values within ± 0.03 pK units over the entire curve. The pK values were calculated for each point on the curve using the method of Sørensen (1909). The values obtained were consistent with those in the literature. DL- α -Alanine of satisfactory purity was obtained after two recrystallizations from aqueous methanol.

Potentiometric titration. A Cambridge Instrument Company bench type pH meter was used with a glass electrode and saturated calomel half-cell. The titrations were carried out as outlined by Albert (1950) with minor modifications. The dissociation constant for the α -amino group was determined under the conditions subsequently used for the titrations in the presence of the metal. These titrations were made on solutions in which the ratio of acid to metal was 2:1 for bivalent and 1:1 for univalent metals. For most titrations equal volumes of stock metal and amino-acid solutions were mixed and then stirred with O_2 -free N_2 for 10 min. At the midpoint of the titration the volume was 50 ml.

Calculations. The two systems to be considered in calculating the stability of each complex are:

- (a) $\text{M}^{++} + \text{amino-acid} = \text{complex I}^+$ with dissociation constant $= K_1$,
- (b) $\text{complex I}^+ + \text{amino-acid} = \text{complex II}$ with dissociation constant $= K_2$,

where M^{++} is a bivalent metal. The overall stability of the final complex $\log K_s$ is given by

$$\log K_s = \log K_1 + \log K_2.$$

The calculation of $\log K_s$ takes account of the proportion of amino-acid in the form available for chelation at a particular pH. This form of the amino-acid is that in which the carboxyl group is ionized and the amino group unionized. All values for $\log K_s$ were calculated from the point at which one molecule of the amino-acid was bound to the metal ion. At this point the following equation was used: $\log K_s = -2 \log [\text{Sc}]$, where $[\text{Sc}]$ is the concentration of free amino-acid available for chelation at this pH (Albert, 1950). The more critical method of calculating values for either $\log K_1$ or $\log K_2$ for each point on the titration curve was also used. These methods yielded values for $\log K_s$ agreeing within 0.1 unit for the amino-acids and 0.2 unit for glycylglycine.

RESULTS

Table 1 shows the $\log K_s$ values obtained for glycine and those of its derivatives in which the hydrogen atoms of the amino group were substituted. There are no values for Mg, Ca, Sr and Ba as these were too low to obtain by the present method. Fig. 1 shows the relative stability constants for glycine with Group IIb metals. Table 2 is a comparison of the stability constants obtained by different authors using mainly potentiometric methods.

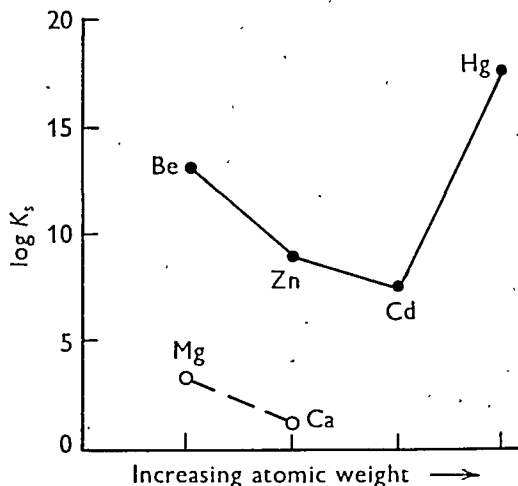


Fig. 1. Typical distribution of stability constants of Group II metals with an amino-acid (in this case glycine). The figures for magnesium and calcium are quoted from Monk (1951c).

DISCUSSION

Potentiometric titrations of α -amino-acids with metals of Group II showed that the two sub-groups behaved differently. The Group IIa metals, barium, strontium and calcium either did not form complexes, or only did so with very low stability constants. In contrast, the Group IIb metals, zinc, cadmium and mercury formed complexes with stability constants which could be readily measured. The other elements of the group behaved in different ways. Beryllium appeared to form stable complexes and was therefore associated with Group IIb. Magnesium, however, formed complexes with low stabilities which could not be measured accurately and it behaved as though it were intermediate in properties between the two groups. Monk (1951c) quoted $\log K_s$ values for calcium which were well beyond the limit measurable by the method used. He also showed that magnesium had $\log K_s$ values of approximately 4.0. This is in agreement with the values of Albert (1950) and those found in the present investigation.

Throughout this investigation it was apparent that the metal was the dominating factor in determining the stability constants of the complexes. This confirmed the observation of Albert (1950) on a wide range of metals. Fig. 1 showing the relative stabilities of the metal complexes with glycine was typical of all the amino-acids studied. The order of stability was $Hg > Be > Zn > Cd$. So far no metal has

possible to assess complex formation on the alkaline side of this pH.

The effect of ligand structure on complex stability was examined. No appreciable effect was found when the hydrogen atom of the glycine amino group was substituted as in sarcosine, proline and hydroxyproline. The values in Table 1 show differences which are not temperature effects, but are slight

Table 1. *Stability constants for metal complexes of α -amino-acids with only two ionizing groups*

(The effects of substituting the hydrogen atoms of the amino group and of carbon chain length and isomerism are shown.)

Amino-acid	Temperature (°)	pK	log K_s			
			Be	Zn	Cd	Hg
Glycine	22.0	9.73	13.3	9.2	7.9	18.2
Glycylglycine	21.0	8.21	9.8	6.4	5.4	12.4
Sarcosine	20.0	10.22	13.9	8.8	7.5	18.7
L-Proline	17.0	10.46	14.2	9.9	8.0	20.5
L-Hydroxyproline	17.0	9.70	12.7	9.6	8.2	17.7
DL- α -Alanine	21.0	9.90	13.1	9.1	7.6	18.4
DL- α -Amino-n-butyric acid	20.0	9.62	12.9	8.3	6.8	18.5
DL-Norvaline	20.0	9.87	12.6	8.1	6.6	17.7
DL-Norleucine	20.0	9.92	12.8	8.5	6.9	17.8
DL- α -Aminoisobutyric acid	19.0	10.24	12.4	8.8	7.2	18.3
DL-Valine (MBT)*	19.5	9.59	12.4	8.2	6.7	†
DL-Isoleucine	20.0	9.86	12.6	8.2	6.6	17.6
DL-Leucine	20.0	9.92	13.2	9.1	7.8	17.5

* Microbiologically tested.

† Insoluble complex.

Table 2. *Comparison of stability constants in the literature of complexes formed with glycine, α -alanine and glycylglycine with various metals*

Amino-acid or peptide	Metal									
	Cu	Ni	Zn	Pb	Co	Mg	Ca	Ag†	Cd	Hg
Glycine	(1) 15.4	11.0	9.3	—	8.9	4.0	—	—	8.1	—
	(2) 15.6*	11.1	10.0	8.9	9.3	3.4†	1.4†	3.5*	—	—
	(3) —	11.0	9.2	9.3	8.8	—	—	3.7	7.9	18.2
	(4) 15.2	10.6	8.9	—	8.4	—	—	3.7	7.1	19.2
	(5) 15.4	—	9.7	—	8.9	—	—	—	—	—
α -Alanine	(1) 15.1	—	—	—	8.4	—	—	—	—	—
	(2) 15.4*	10.7	9.5	8.2	8.5	2.0†	1.2†	3.6*	—	—
	(3) —	—	9.4	—	—	—	—	—	7.2	—
Glycylglycine	(2) 11.7*	7.9	6.6	6.0	5.9	1.1†	1.2†	2.7*	—	—
	(3) —	7.6	6.4	5.8	5.8	—	—	3.1	5.4	12.4

(1) Albert (1950). (2) Monk (1951a, b, c). (3) Present work. (4) Flood & Loras (1945). (5) Maley & Mellor (1950).

* Solubility studies.

† First constant only.

been quoted in the literature as showing greater complex stability than mercury. The anomalous increase in stability when passing from cadmium to mercury was in accord with the work of Bjerrum (1941) on metal ammine formation. Experiments using mercuric chloride gave fallacious low results due to the mainly covalent nature of this salt. The ionized mercuric nitrate, however, gave the high values recorded here. The values for beryllium must be treated with caution as both its salts and complexes hydrolyse above pH 6. This made it im-

possible to assess complex formation on the alkaline side of this pH.

The substitution of the α -amino hydrogen atom by another amino-acid as in glycylglycine, however, produced a decrease in stability comparable to the metal effect.

In Table 2 a comparison is made, between the results of a number of workers. In general, good agreement was obtained for glycine and α -alanine. The purpose of this part of the work was to check the results from glass-electrode measurements with those of other authors and with the solubility measurements of Monk (1951c). Monk (1951a)

had already shown that solubility and titration measurement gave comparable results.

The results for glycylglycine agreed well with those of Monk (1951c). They were all measured at pH more acid than 8.0. At more alkaline pH values Dobbie, Kermack & Lees (1951) have shown that with cupric ions the peptide nitrogen also takes part in chelation. Evidence of this further chelation was obtained with mercury in this study. The titration curve obtained was symmetrical and yielded satisfactory results for $\log K_1$ and $\log K_2$.

The marked decrease in stabilities from glycine to glycylglycine is of interest, as according to Albert (1950) the metal bridges the carboxyl and amino groups of glycine. In the case of glycylglycine such ring formation is unlikely in view of the size of such a ring. As, however, there is evidence for the peptide losing a second proton per molecule (Dobbie *et al.* 1951) it is more probable that the metal chelates first with the terminal amino groups and then with the peptide nitrogen. Further work on metal peptide complexes is in progress.

The effect of lengthening the chain length on stability constants was small, but produced a still detectable decrease in stability from glycine to norvaline (Table 1). Norleucine, however, gave an unexpected increase in stability. The effect of isomerism in the chain was also studied. From Table 1

it will be seen that the isomers of norleucine gave different stability constants but the isomers of norvaline gave identical values.

SUMMARY

1. The stability constants of some complexes of α -amino-acids with metals of Group II of the periodic classification were measured by a potentiometric method.

2. The predominant part played by the metal in determining the stability constant was confirmed.

3. Metals of Group IIa formed complexes with stability constants too low to be measured. Group IIb metal complexes yielded measurable constants in the order of magnitude $\text{Hg} > \text{Be} > \text{Zn} > \text{Cd}$. The results of a number of investigators using a range of metals with glycine, α -alanine and glycylglycine were compared.

4. The effect of substituting the hydrogen atoms of the amino group of glycine on stability constants was studied. The only appreciable structural effect observed was the decrease in stability produced by peptide formation. Increase of chain length resulted in decreased stabilities.

I wish to thank Dr N. H. Martin for his advice and encouragement during the course of this work.

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The Crystallization of Fumarase

By V. MASSEY

Department of Biochemistry, University of Cambridge

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Fumarase, the enzyme catalysing the reversible hydration of fumaric acid to L-malic acid, was discovered in 1911 by Batelli & Stern. Since then it has been shown to occur in a large number of plant and animal tissues, and in lower organisms.

In 1941 Laki & Laki claimed to have isolated crystalline fumarase from pig-heart muscle, but Scott (1948), Scott & Powell (1948) and Racker (1950) have shown this preparation to contain considerable quantities of aconitase and lactic de-

hydrogenase. Scott (1948) has also obtained amorphous fractions with over three times the specific activity of the preparation of Laki & Laki. In this communication is reported the isolation from pig-heart muscle of crystalline fumarase; homogeneous by sedimentation and electrophoretic criteria, which has a specific activity almost ten times that reported by Laki & Laki. A preliminary account of this work has been published (Massey, 1951).

METHODS

Enzyme activity determinations. Fumarase activity was determined by the spectrophotometric method of Racker (1950). The reaction was measured in the presence of 0.017M-sodium fumarate and 0.033M-phosphate buffer, pH 7.3, using a Beckman model DU spectrophotometer. One unit of activity was chosen arbitrarily as the amount of enzyme required to reduce the optical density of fumaric acid at 3000 Å. by 0.01/min. at 20° and pH 7.3. The initial rate of reaction was calculated by plotting a graph of optical density against time of reaction.

Protein concentration. This was measured by Kjeldahl nitrogen estimations on dialysed extracts. In all calculations the protein concentration was assumed to be 6.25 times the nitrogen content. Approximate concentrations were also obtained by optical density determinations at 2770 Å. The optical density of 1 ml. of protein solution in a final volume of 3 ml., determined through a quartz cell of 1 cm. thickness was referred to as density/ml. (*d*/ml.).

Sedimentation and diffusion. The sedimentation constant was determined using a Svedberg oil-turbine ultracentrifuge and the diffusion constant using a Gouy diffusimeter (see Addendum).

Electrophoresis. Electrophoretic mobilities were determined with a Perkin Elmer electrophoresis apparatus at 1°. The current passed was 8 ma.

EXPERIMENTAL

Method of isolation

Fresh pigs' hearts were cleaned of fat and connective tissue, minced, and washed with large quantities of tap water until the washings were almost colourless. The washed mince was squeezed in cheesecloth, and homogenized with three times its weight of cold 0.01M- Na_2HPO_4 for 3 min. The homogenate was spun for 30 min. at 1800 g in an International Serum Centrifuge, and the supernatant poured from the residue. The pH of the supernatant was adjusted to 5.2 with 1M-acetate buffer, and the inactive precipitate centrifuged off. The clear, slightly reddish supernatant contained fumarase in almost the same quantities as the supernatant before adjusting the pH to 5.2, but the protein concentration was reduced to 10–14% of the initial value. The fumarase was then adsorbed quantitatively on $\text{Ca}_3(\text{PO}_4)_2$ gel. The amount necessary varies from preparation to preparation, and must be determined by pilot lots for maximum yield of enzyme. It is generally in the range of 4–7 ml. of gel (containing 30 mg. dry wt./ml.) for 100 ml. of solution. The $\text{Ca}_3(\text{PO}_4)_2$ was centrifuged and the supernatant discarded. The enzyme was next eluted from the gel with cold 0.1M-phosphate buffer, pH 7.3, containing 50 g. $(\text{NH}_4)_2\text{SO}_4$ /l. The fumarase can be eluted almost quantitatively from the gel—three to four elutions are sufficient to remove the bulk of the enzyme. The combined eluates were then fractionated between 45 and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$, the 45–60% fraction containing the enzyme. This fraction was dissolved in distilled water, and dialysed overnight against distilled water. Considerable purification was then achieved by the addition of small amounts of $\text{Ca}_3(\text{PO}_4)_2$ to this concentrated protein solution. Sufficient $\text{Ca}_3(\text{PO}_4)_2$ was added to combine with impurities, but not sufficient to adsorb fumarase. The quantities vary from one preparation to

another, and are best determined by the addition of gradually increasing amounts of gel, followed each time by centrifugation and activity and protein determinations. This was followed by another $(\text{NH}_4)_2\text{SO}_4$ fractionation, the precipitate formed on the addition of $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation was centrifuged at high speed and dissolved in the minimum amount of 15% saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Saturated $(\text{NH}_4)_2\text{SO}_4$ was added drop by drop until a faint turbidity was formed. The solutions of $(\text{NH}_4)_2\text{SO}_4$, which were of A.R. grade, had not been neutralized. The pH of the mixture before crystallization was between 6.1 and 6.4. After two days in the refrigerator, crystals formed in large amounts (Fig. 1). These are relatively insoluble in water; they can be washed quickly and centrifuged without much loss through solution.

Recrystallization. Fumarase crystals were centrifuged, washed with cold distilled water, centrifuged again, and dissolved in 0.01M-phosphate buffer, pH 7.3. From this, the enzyme was precipitated by 60% saturation with $(\text{NH}_4)_2\text{SO}_4$, dissolved in 15% saturated $(\text{NH}_4)_2\text{SO}_4$, and saturated $(\text{NH}_4)_2\text{SO}_4$ added to produce turbidity as in the original crystallization. Crystallization can be greatly accelerated by seeding from a previous preparation. After two recrystallizations the preparation reaches a constant specific activity, which is from 2000 to 3000 times that of the original extract. With care, the overall yield can be 40–50%. The activity and purity data for a representative preparation are shown in Table 1.

Recrystallization from phosphate buffer, pH 6.3. Fumarase can also be obtained in crystalline form by dialysis against 0.1M-phosphate buffer, pH 6.3. Fumarase crystals from $(\text{NH}_4)_2\text{SO}_4$ were dissolved in 0.01M-phosphate buffer, pH 7.3, and the enzyme precipitated in amorphous form by 60% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in 0.1M-phosphate, pH 7.3, and the pH gradually lowered by dialysis against 0.1M-phosphate, pH 6.3.

The enzyme crystallized in the form of thin rectangular plates (Fig. 2). These crystals are almost insoluble in water and dilute phosphate solutions, but can be dissolved in dilute NaOH solution. If this is done at 0° and the pH adjusted to neutrality as soon as the crystals are dissolved, there is no loss in activity. The solution so obtained has the same specific activity as solutions of fumarase obtained from $(\text{NH}_4)_2\text{SO}_4$. All the other results reported in this paper however have been obtained with the crystals from $(\text{NH}_4)_2\text{SO}_4$.

Pig-heart protein A. From the mother liquor of the crystallization of fumarase, another crystalline protein can be obtained on the addition of a little more $(\text{NH}_4)_2\text{SO}_4$. Here crystallization proceeds best at 20–25°. The crystals, unlike those of fumarase, are very soluble in water, and appear as long thin needles without sharply defined edges. Attempts to identify the crystals have not yet been successful.

Properties of fumarase

Like other crystalline enzymes, fumarase is protein in nature; it gives a strong biuret reaction, is denatured by heating, and by extremes of acidity and alkalinity. It is reasonably stable over the pH range 5–9. The crystals are faintly birefringent in polarized light, and do not contain any ammonium sulphate. They are slightly soluble in distilled water, but readily soluble in 0.01M-phosphate

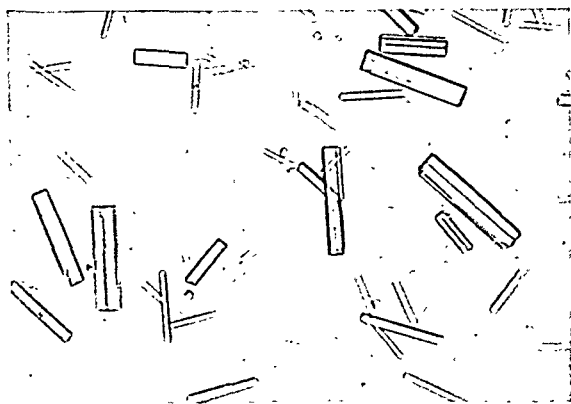
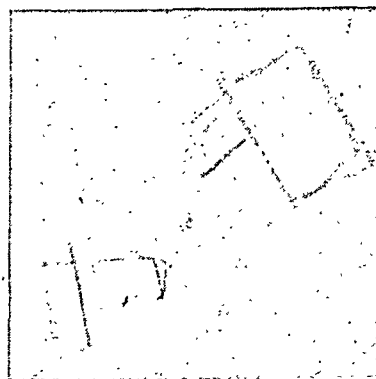
Table 1. *Details of a representative preparation of fumarase*

Treatment	Activity (units/ml.)	Protein concn. (d/ml.)*	Purity (units/d)	Volume (ml.)	Total units	Times purified	Yield (%)
Pigs' hearts homogenized with 0.01 M- Na ₂ HPO ₄ . Supernatant	9	6.53	1.37	4800	43000	—	—
Adjusted to pH 5.2. Supernatant	8.5	0.45	19	4750	40050	14	94
Added 185 ml. Ca ₃ (PO ₄) ₂ gel (30 mg./ml.). Supernatant	0	—	—	—	—	—	—
Eluted four times with 0.1 M-phosphate, pH 7.3, containing 50 g. (NH ₄) ₂ SO ₄ /l.	66	1.35	49	590	39000	36	91
Fractionated between 45–60% saturation with (NH ₄) ₂ SO ₄	1050	5.85	180	30	31500	132	73
Dialysed overnight against distilled water	750	3.90	192	42	31500	140	73
Added Ca ₃ (PO ₄) ₂ gel. Supernatant from							
0.5 ml.	750	3.70	203	42	31500	148	73
1.0 ml.	750	3.15	238	42	31500	174	73
2.5 ml.	700	2.45	286	43	30000	209	70
5.0 ml.	679	1.85	362	45	30000	265	70
10.0 ml.	510	0.85	600	50	25500	438	59
Added (NH ₄) ₂ SO ₄ to 60% saturation, precipitate dissolved in 15% sat. (NH ₄) ₂ SO ₄ . Crystallized	—	—	—	—	—	—	—
Crystals dissolved in 0.01 M-phosphate buffer, pH 7.3	1200	0.48	2500	15.1	18100	1830	42
1st Recrystallization	1800	0.62	2900	9.5	17100	2110	40
2nd Recrystallization	1560	0.54	2900	10.5	16400	2110	38

* For definition of d/ml. see Methods.

buffer, pH 7.3. At pH 7.3 and 20°, in the presence of 0.033 M-phosphate, 1 g. of crystals is capable of converting 0.55 mole (i.e. 64 g.) of fumaric acid to

& Laki (1941), the activity was 1.30 moles of fumaric acid converted to L-malic acid/min./g. enzyme. The activity reported by Laki & Laki (1941) was 0.14 mole/min./g. enzyme.

Fig. 1. Fumarase crystals from ammonium sulphate.
× 600.Fig. 2. Fumarase crystals from 0.1 M-phosphate, pH 6.3.
× 950.

L-malic acid/min. On the basis of a molecular weight of about 200 000, as indicated by sedimentation and diffusion, this represents a turnover number of over 100 000 moles substrate/min./mole fumarase, under the above conditions.

At pH 6.7 and 39° in the presence of 0.1 M-phosphate, the conditions which were used by Laki

As indicated by previous studies with partially purified preparations, crystalline fumarase has a very high specificity, attacking only fumaric and L-malic acids. Maleic, D-malic, *trans*- and *cis*-aconitic, tartaric, aspartic and crotonic acids are completely unattacked by even large concentrations of fumarase.

Molecular weight. The molecular weight was determined by sedimentation and diffusion measurements of a 0.7% (w/v) solution of fumarase in 0.087M-phosphate buffer of pH 7.34. The results, which are given in the Addendum, show a molecular weight of 204 000 under these conditions, assuming a partial specific volume of 0.75.

and (d) show the initial position of the boundary and are the usual boundary anomalies found with high protein concentrations.

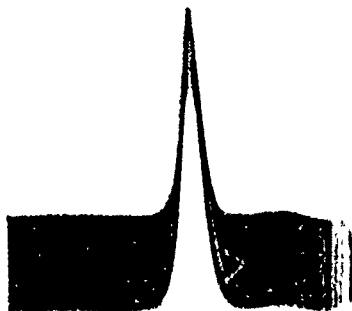
From the mobilities at these pH values, an extrapolated value for the isoelectric point of fumarase is between pH 5.0 and 5.4. Unfortunately, the electrophoretic behaviour of fumarase at pH values lower



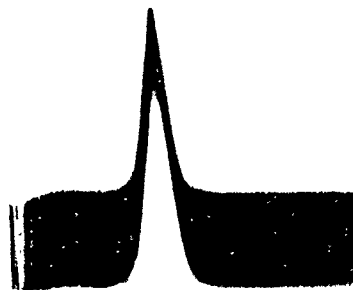
(a) pH 5.8. Time after beginning of exp. 5520 sec. Ascending boundary.



(b) pH 5.8. Time, 5520 sec. Descending boundary.



(c) pH 7.34. Time, 8360 sec. Ascending boundary.



(d) pH 8.1. Time, 7875 sec. Descending boundary.

Fig. 3. Electrophoresis schlieren diagrams.

Electrophoretic behaviour. Electrophoresis was performed at pH 8.1, 7.34 and 5.8, at a constant ionic strength of 0.21. At these pH values the

than 5.8 could not be studied, because of the low solubility in this pH region. However, the extrapolated value agrees well with the isoionic point of pH 5.0 as determined by exhaustive dialysis of a salt-free solution of fumarase.

Table 2. Electrophoretic mobilities of fumarase

pH	Mobility (cm. ² sec. ⁻¹ V. ⁻¹ × 10 ⁻⁵)
5.8	0.93
7.34	2.25
8.1	2.45

material appeared to be perfectly homogeneous. The results are shown in Fig. 3 and Table 2. The slight bumps, away from the main peaks, in Fig. 3 (c)

SUMMARY

1. The isolation of crystalline fumarase and another, as yet unidentified, crystalline protein from pig heart is described.

2. Sedimentation and diffusion constants indicate that fumarase is a homogeneous protein of molecular weight about 200 000.

3. Electrophoresis studies at three pH values indicate a homogeneous protein. The results give an isoelectric point for fumarase between pH values of 5.0 and 5.4.

4. The turnover number of fumarase is over 100 000 at 20° and pH 7.3 in the presence of 0.033 M-phosphate.

The principle of the initial stages in the purification was based on results obtained in an advanced class experiment under the direction of Dr M. Dixon, F.R.S., Dr S. J. Bach

and Dr E. C. Webb at the Biochemistry Department, Cambridge. I am indebted to Dr R. Cecil and Dr A. G. Ogston of the Biochemistry Department, Oxford, for the determination of sedimentation and diffusion constants, and to Dr B. Conway and Mr D. W. F. James of the Chester Beatty Research Institute, London, for electrophoretic mobility measurements. I wish to thank Dr M. Dixon for suggesting the problem and for his advice and help in this work. The work was carried out with a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

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ADDENDUM

Sedimentation and Diffusion

BY R. CECIL AND A. G. OGSTON
Department of Biochemistry, Oxford

(Received 16 October 1951)

A solution of fumarase containing 0.7 g./100 ml., dialysed against a buffer containing 0.067 M- Na_2HPO_4 and 0.017 M- KH_2PO_4 , was examined in the Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948) and in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948).

A symmetrical boundary curve was obtained in the ultracentrifuge, whose area represented 96 % of the refractive increment of the solution, measured against diffusate. The sedimentation constant, corrected to its value in water at 20°, was 8.51×10^{-13} .

The mean diffusion constant, corrected to its value in water at 20°, was 4.45×10^{-7} . Analysis of the Gouy interference pattern (Ogston, 1949) showed

that the material was not homogeneous, but contained 7 % of rapidly diffusing material; the diffusion constant of the main component was 4.05×10^{-7} . The proportions of the main component estimated by the ultracentrifuge (96 %) and by diffusion (93 %) agree within the accuracy of these estimates. The presence of some rapidly diffusing material might have been due to incomplete dialysis.

Using the corrected value for the diffusion constant of the main component and assuming a value of 0.75 for the partial specific volume, a value of 204 000 is obtained for the molecular weight. Although this is derived from quantities measured at a single concentration, it is not likely to differ much from the true value.

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The Bound Nucleotide of the Isolated Myofibril

By S. V. PERRY

Biochemical Laboratory, University of Cambridge

(Received 22 November 1951)

Since Straub & Feuer (1950) reported the presence of adenosinetriphosphate (ATP) in globular actin (G-actin) the nucleotide content of this protein has been the subject of considerable investigation. The original claim that ATP was split during the transformation of G-actin to fibrous actin (F-actin) has been substantiated by the work of Laki & Clark (1951) and Szent-Gyorgyi (1951), but the work of these authors does not support the view that depolymerization is accompanied by resynthesis of ATP. On the other hand, Dubuisson & Mathieu (1950), whilst confirming that ATP is present in G-actin preparations, could not detect any change in phosphate distribution or any change in free phosphate during polymerization. Although the above investigators do not agree as to the role of ATP in the polymerization of actin, all confirm that ATP is a component of normal G-actin preparations, whereas Snellman & Gelotte (1951), using chromatographic techniques, were unable to detect any ATP or adenosinediphosphate (ADP) in either G-actin or F-actin. These authors consider that the prosthetic group of actin is a dinucleotide containing adenine and a so-far unidentified base which can occur in phosphate rich and phosphate poor forms, corresponding to G-actin and F-actin respectively.

Actin is not alone in its property of binding adenine nucleotides, for Buchtal, Deutsch, Knappeis & Munch-Petersen (1949) have shown that when myosin and actomyosin are treated with ATP, some nucleotide is bound to the protein in such a way that it cannot be removed by repeated washing.

The investigations so far mentioned have been carried out on extracted purified myofibrillar proteins and the nucleotide content of the intact myofibril has received little attention. Using ultra-violet absorption methods Caspersson & Thorell (1942) claimed to have demonstrated the localization of ATP in the I band of resting muscle. It is not possible, however, to decide whether their results were due to adenine nucleotide or to the small amount of nucleic acid which occurs in the myofibril (Perry, 1952). In an earlier communication (Perry, 1951) a method employing collagenase was described for the isolation of myofibrils in comparatively small quantities. Preliminary analyses of these preparations indicated that the iso-

lated myofibril contained small amounts of nucleic acid, phospholipid and acid-labile phosphorus. In the present paper a study of this latter phosphorus fraction is described, together with a method for isolating myofibrils in larger quantities which has considerably facilitated the investigation. The acid-labile phosphorus of myofibrils obtained from skeletal and cardiac muscle of the rabbit, and rat skeletal muscle, is rather constant in amount. In rabbit skeletal muscle it arises from ATP and ADP bound to the myofibril in a manner which renders these nucleotides inaccessible to the enzymes which normally attack them. This nucleotide fraction consists predominantly of ADP, and the quantities present are compatible with the view that it is associated with F-actin in the isolated myofibril.

METHODS

Preparation of myofibrils. Muscle from the hind legs and back of a rabbit was quickly dissected out, chilled in ice and minced. The mince was homogenized in a Waring Blendor for 2 min. with 8 vol. of 0.08M-borate buffer, pH 7.1. This and all subsequent operations were carried out at 0°. Centrifugation of the homogenate for 15 min. at 600 g was usually sufficient at this stage to give a compact sediment which was resuspended in the same volume of borate buffer as was used to make the original homogenate. The suspension was homogenized again for 2 min. This homogenate was centrifuged for 20 min. at 600 g, the turbid supernatant discarded and the lighter-coloured upper layer of the sediment carefully removed with the aid of a little borate buffer. This upper layer, which consisted mainly of myofibrils, was freed from coarser material by centrifugation for 5 min. at 300 g.

The myofibrils were washed free from granules (Perry, 1952) and soluble sarcoplasmic components by repeated resuspension in borate buffer followed by centrifugation for 20–30 min. at 600 g. To obtain a suspension of myofibrils which contained less than 2% of protein in solution, 4–5 centrifugations were necessary. These centrifugations were carried out as rapidly as possible because, after standing some hours in borate buffer, the myofibrils became increasingly difficult to centrifuge down at 600 g. It is desirable to keep the centrifugal field at this figure to ensure a satisfactory separation of granules and myofibrils. After an additional centrifugation at 300 g for 5 min. to remove any remaining large particles and myofibrils which may have denatured and clumped, the preparation was stored at 0° in the presence of a trace of toluene. The stored suspension was very viscous and usually contained 10–15 mg. protein per ml. By this method 5–10 g. of myofibrils could readily be

obtained from 200 g. of minced muscle. When examined microscopically with the aid of methylene-blue stain the preparations could be seen to be free from other components.

Isolation of nucleotide preparation. Myofibrils were washed twice by centrifugation and resuspension in distilled water to remove most of the borate buffer and the free phosphate which leaches out of these structures on standing. One volume of cold 60% (w/v) trichloroacetic acid (TCA) was well stirred into 11 vol. of the myofibril suspension. After standing for 20 min. at 0° the fibrous myofibrillar

by following the fall in absorption at 265 m μ . when the nucleotide was treated with (1) adenylic 5-deaminase, (2) myokinase and adenylic 5-deaminase, (3) potato pyrophosphatase and adenylic 5-deaminase. The enzyme preparations and the experimental procedures were carried out as described by Kalckar (1947).

Acid-labile P. The inorganic phosphate liberated when the TCA extract of the myofibrils, or an aqueous solution of the nucleotide preparation, were heated with N-HCl for 10 min. at 100°, was estimated by the method of Fiske & Subbarow (1925).

Table 1. *Acid-labile phosphorus content of isolated myofibrils*

Preparation	Condition	Source	Acid-labile P (μ g./g. myofibril)
9/5	Myofibrils stored at 0°:		
	5 days	Rabbit skeletal	111
	26 days	Rabbit skeletal	82
5/6	Myofibrils stored at 0°:		
	1 day	Rabbit skeletal	121
	7 days	Rabbit skeletal	121
	9 days	Rabbit skeletal	100
	24 days	Rabbit skeletal	100
	Treated 2 days with 0.5M-KCl	Rabbit skeletal	83
	Dialysed 2 days against 0.5M-KCl	Rabbit skeletal	63
7/8	Freshly prepared	Rabbit psoas	108
3/8	Muscle kept 3 days at 0° (rigor)	Rabbit psoas	114
17/10	Freshly prepared	Rat hind leg	116
24/10	Freshly prepared	Rat hind leg	92
18/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	146
31/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	100
24/10	Freshly prepared	Rabbit heart	88
11/10	Freshly prepared	Rabbit heart	88
31/10	Muscle stored 4 days at 0° (rigor)	Rabbit heart	111

precipitate was centrifuged down and the supernatant filtered. When the clear filtrate had been extracted four times with ether to remove the bulk of the TCA, the aqueous layer was freed from ether by distillation *in vacuo* in the cold and finally freeze-dried.

The dry, white residue consisting mainly of borate, which acted as a convenient carrier for the nucleotide, was extracted three times with ether to remove the last traces of TCA, and then dried and stored in a vacuum desiccator at 0°. All stages of this preparation were carried out in the cold to avoid the breakdown of acid-labile phosphate. The efficacy of these precautions was shown by the fact that the ratios of free to acid-labile P in the original TCA extract and the final nucleotide preparation were identical.

Enzymic analyses. Myosin, freed from myokinase by repeated precipitation, was prepared by the method of Bailey (1942), and myokinase as described by Colowick & Kalckar (1943). Nucleotide preparation corresponding to 10–20 μ g. of acid-labile P was incubated with the enzymes individually, and then with both together, in the presence of 0.1M-glycine buffer, pH 9.0, and 0.005M-CaCl₂; final vol. 2 ml. Incubations were carried out for 30 min. at 33°. Extra enzyme added at the end of this period induced no further splitting of phosphate, indicating that the reaction had gone to completion.

Spectrophotometric analysis for adenylic acid, ADP and ATP was carried out with the Beckman spectrophotometer

RESULTS

TCA extracts of myofibrils from fresh rat and rabbit skeletal muscle contained acid-labile P ranging in amount from 90 to 140 μ g./g. of myofibril (the dry weight of the myofibril was taken as six times the nitrogen content). Values within this range were also obtained from muscle (rabbit psoas and rat hind-leg) which had been allowed to go into rigor by storing at 0°.

Continued washing with 0.08M-borate, pH 7.1, did not remove the acid-labile P from the myofibrils; after storing the suspensions in this buffer for 3–4 weeks at 0° the amount had fallen by only 20–30%. During storage the acid-labile P remained bound to the myofibril and no significant amount could be detected in the clear supernatant obtained by centrifuging the suspension for 10 min. at 7000 g. Table 1 summarizes these results and also indicates that treatment with M-potassium chloride, which rendered 75–85% of the total protein of the myofibril soluble, only slightly reduced the acid-labile P level. A rather greater decrease was obtained in one experiment when a 0.5M-potassium

chloride extract of the myofibrils was dialysed against 0.5M-potassium chloride for 2 days at 0°.

Experiments with heart muscle indicated that the acid-labile P level is similar to that obtained with skeletal myofibrils. It should be noted, however, that preparations from heart were not usually as satisfactory as those from skeletal muscle, because cardiac tissue does not break down so readily into single myofibrils and the latter are not so easily separated from granules.

Fig. 1 is the ultraviolet absorption curve given by a preparation of nucleotide from well washed skeletal muscle myofibrils. The curve shows a maximum at 259 mμ. and is in general similar to that given by the adenosine polyphosphates. For a

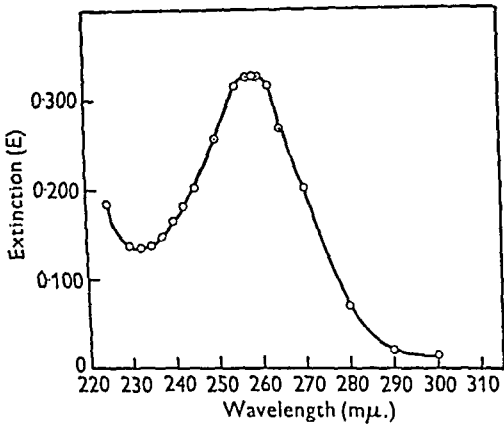


Fig. 1. Ultraviolet absorption of nucleotide from washed muscle myofibrils.

number of reasons it seemed likely that ADP or ATP could be responsible for the acid-labile P of the myofibril; to determine whether this was the case, and if so to estimate the amounts of the two polyphosphates, the nucleotide preparation was analysed by determining the amount of phosphate split from it by myosin, and then by myosin in the presence of myokinase. Table 2 shows that myosin alone split only a fraction of the acid-labile P whereas with myokinase 75-90% was liberated as inorganic phosphate. From these experiments it can be concluded that the acid-labile P arises from a mixture of ATP and ADP, with the latter making up about 80% of the whole. In no case was the total acid-labile P split by these enzymes acting in combination. This does not necessarily mean that other compounds containing acid-labile P are present, for the experience of other workers (cf. Bailey, 1949) with myosin and myokinase acting in combination indicates that it is extremely difficult to get complete hydrolysis of the acid-labile P even with the purest samples of ATP and ADP.

Potato pyrophosphatase prepared by the method of Kalckar (1947) liberated rather more than the

acid-labile P from the nucleotide. On prolonged incubation in the presence of large amounts of enzyme, phosphorus was split from the nucleotide in amounts which approached the total organic P content. Under comparable conditions, strangely enough, the enzyme split only the acid-labile P of an ATP preparation, though it did slowly attack adenylic acid. Bailey (1949) and Krishnan (1949) have pointed out that there is some phosphatase (or

Table 2. Analysis of nucleotide preparation using myosin and myokinase

(Results expressed in μg./g. of nucleotide preparation.)

Nucleotide preparation	N2	N3
Free P	503	1213
Acid-labile P	831	1395
Total organic P	—	2762
Total organic P	—	1.98
Acid-labile P	—	—
Acid-labile P calculated from amounts of ATP and ADP determined spectrophotometrically	770	1485
P liberated by myosin	107	179
P liberated by myosin and myokinase	604	1168
Acid-labile P due to ATP	214	358
Acid-labile P due to ADP	390	810
Moles ADP as % moles ADP plus ATP	79	82

nucleotidase) associated with the Kalckar potato pyrophosphatase preparation. If the pyrophosphatase is used in low concentration and particularly if the adenylic acid is removed as it is formed, as in the Kalckar spectrophotometric estimation of the adenine nucleotides, interference by the phosphatase activity is small.

Table 3. Relative proportions of adenosine phosphates in nucleotide fraction isolated from rabbit myofibrils

(Figures for each preparation represent the average of two or more estimations.)

Nucleotide preparation	Age of myofibril preparation (days)	(Moles % total moles adenine nucleotides)		
		Adenylic acid	ADP	ATP
N2	10	17	75	8
N3	1	13	79	8
Average		15	77	8

In Table 3 are shown the results of enzymic analyses for the adenosine phosphates carried out by the method of Kalckar (1947). Although these estimations indicate a higher relative proportion of ADP than was obtained by assay with myosin and myokinase, the results given by the two methods are in satisfactory agreement, considering their accuracy (Table 2).

The work of Buchtal *et al.* (1949), on the adsorption of adenine nucleotides on myosin and actomyosin, suggests that during the preparation of

myofibrils, nucleotides could be picked up from solution and adsorbed on these structures in a similar way. The rather constant composition and amount of the nucleotide fraction associated with the myofibrils makes such an explanation unlikely, a view which is supported by the following experiment. ATP was added to myofibril suspensions in 0.08M-borate buffer, pH 7.1, so that the final concentrations of acid-labile P were 10 and 100 times that bound to the myofibrils. At the higher ATP concentration the myofibrils had very obviously synaerased, but in all cases after washing by centrifugation, and resuspension in the borate buffer, there was no significant difference between the acid-labile P content of the control myofibrils and those treated with ATP. For (a) control myofibrils, (b) myofibrils treated with ATP equivalent to 10 times, and (c) myofibrils treated with ATP equivalent to 100 times their acid-labile P content, the results were 127, 143 and 131 μg . of acid-labile P/g. respectively.

the myofibril. The ultraviolet absorption spectrum of the nucleotide preparation did not show any evidence of a maximum at 250 $\text{m}\mu$., the position of maximum absorption by inosinic acid.

The fact that the acid-labile P is not split off from the myofibrils when muscle passes into rigor probably accounts, in part at least, for the residue of easily hydrolysable phosphate reported by Bendall (1951) to be present in rigor muscle.

The amounts and relative proportions of adenylic acid, ADP and ATP are similar to those which would be expected if all the nucleotides were considered to be associated with actin in the fibrous form in the intact myofibril. If actin makes up 20 % of the myofibrillar protein (cf. Hasselbach & Schneider, 1951), the actin present in the myofibril would have a nucleotide content which is similar to that of the extracted F-actin analysed by Szent-Gyorgyi (1951). It is a matter of some interest to decide whether actin exists in the globular or fibrous form in the living cell. To assess the true significance

Table 4. *Nucleotide content of actin and isolated myofibrils*

(Results expressed as $\mu\text{moles/g.}$)

Materia	Adenylic acid	ADP	ATP	Authority
Myofibrils*	0.88	2.7	0.47	—
Actin in myofibrils†	4.4	13.5	2.3	—
F-actin	—	—	8.5	Laki, Bowen & Clark (1950)
F-actin	1.6	11.7	2.4	Szent-Gyorgyi (1951)
G-actin	—	—	3.4	Laki <i>et al.</i> (1950)
G-actin	—	—	9.8	Laki <i>et al.</i> (1950)
G-actin	—	—	23	Straub & Feuer (1950)
G-actin	—	—	12-39	Dubuisson & Mathieu (1950)
G-actin	2.1	1.9	7.8	Szent-Gyorgyi (1951)

* These values are obtained by taking 5.9 as the molar ratio of ADP to ATP (average of the results obtained by the two methods described) in rabbit skeletal myofibrils containing 113 μg . of acid-labile P/g.

† Calculation made on the basis that all the nucleotide is associated with the actin which makes up 20 % of the total myofibrillar proteins.

DISCUSSION

From the evidence presented it would appear that the nucleotides associated with the myofibrils are bound to its structure in such a way that although the system contains myokinase and an active adenosinetriphosphatase (Perry, 1951) they are not attacked by these enzymes; that is, so far as can be judged by the maintenance of the acid-labile P level in stored myofibrils. In this respect the bound ADP of the myofibrils showed some differences from that associated with F-actin, for Laki & Clark (1951) have reported that a short time after the addition of salt to G-actin, practically no ADP could be found. This effect they attributed to the myokinase activity of their actin preparations.

In addition to ADP and ATP, adenylic acid is also present and this compound also must be unavailable to adenylic deaminase which is present in

of the state of the nucleotide in the isolated myofibril it must be known whether the system is in the relaxed or the resting condition because it is possible, although it has yet to be proved, that contraction is accompanied by a change in the state of polymerization of actin. Electron microscope examinations (kindly carried out by Mr R. W. Horne of the Cavendish Laboratory) of myofibrils isolated by the method described in this paper show the I band to be shorter than the A band, suggesting that at some stage of the preparation a certain degree of contraction had taken place. It must be concluded then that the actin in the isolated myofibril corresponds to the contracted or partly contracted state.

In Table 4 the nucleotide analyses of actin carried out by various authors are presented. The considerable variations in these values probably reduce their reliability, and they should be compared with the rather constant values for the acid-labile P of

isolated myofibrils as shown in Table 1. Many of the actin preparations have higher ATP values than might be expected in view of the low amount of this nucleotide in the well washed muscle residue from which actin is extracted, and which must consist mainly of myofibril residues. Straub & Feuer (1950) found insignificant amounts of ATP in the acetone-dried fibre from which they extracted G-actin and concluded that ATP was produced during the water extraction of the fibre.

Assuming that the adenine content of the isolated myofibril is compatible with that found *in vivo* it is possible to speculate on the ATP distribution within the muscle cell. Normal muscle contains powerful ADP phosphorylating systems and it is likely that in the resting state all the adenine exists as ATP. This would mean that about 10% of the ATP content of the cell is associated with the myofibrils. As the latter occupy about two-thirds of the cell volume, the concentration of ATP in the extra-myofibrillar space, which is occupied by the sarcoplasm, will be 18 times that in the myofibril, assuming that the myofibrils are not interpen-

trated by the sarcoplasm, or alternatively that any ATP which does penetrate into them is broken down. This concentration gradient must be an important factor affecting the accessibility of ATP to the enzyme associated with the contractile structure, the myosin adenosinetriphosphatase.

SUMMARY

1. A non-enzymic method for the isolation of myofibrils from rabbit skeletal muscle has been described.

2. Myofibrils from cardiac and skeletal muscle of the rabbit and skeletal muscle of the rat contained 90–140 μ g. of acid-labile phosphorus per gram. Similar values were obtained with rigor muscle.

3. The acid-labile phosphorus of myofibrils isolated from rabbit muscle arises from adenosine-diphosphate, and to a lesser extent, adenosinetriphosphate. These nucleotides are bound to the myofibril in such a way that they are not acted upon by the enzymes occurring in myofibril which normally attack them.

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Histidine α -Deaminase and the Production of Urocanic Acid in the Mammal

By D. A. HALL

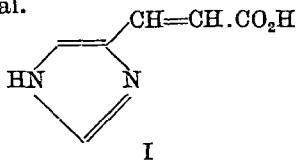
Department of Medicine, University of Leeds

(Received 23 October 1951)

Urocanic acid (β -4(5)-glyoxalinylacrylic acid) (I), as discovered by Jaffe (1874) in the urine of one dog and was considered after its rediscovery by Siegfried (1898) to be a possible normal breakdown product of histidine. Evidence produced by Knistrick (1917, 1919) showing that it was formed from histidine by the action of certain micro-

organisms, and the determination of its structure by Hunter (1912) fostered the suggestion that direct deamination played a part in the normal degradation of histidine. This point of view was supported by the later work of Kotake & Konishi (1922), Kiyokawa (1933) and Harrow & Sherwin (1926). Cox & Rose (1926), however, and Darby & Lewis

(1942), came to the conclusion that the animals, in the urine of which urocanic acid had been observed, were abnormal.



Edlbacher and his co-workers (see review by Edlbacher, 1943) isolated from liver two enzymes which might play an important part in the breakdown of histidine. One of these, to which he gave the name histidase, degrades histidine to glutamic acid, formic acid and ammonia by fission of the iminazole ring. The other, urocanase, performs a similar function on urocanic acid. Edlbacher did not prove the existence of an enzyme capable of directly removing the amino group from histidine; and although he published a scheme showing the possibility of two distinct pathways for histidine breakdown, he considered that his evidence precluded the existence of a path requiring the intermediary production of urocanic acid.

The gravimetric methods whereby urocanic acid was first isolated were only applicable to the considerable quantities available in large volumes of urine, and could not be used easily on liver tissue or extracts. The method employed by Edlbacher & Bidder (1942), although more sensitive, could not be used to identify urocanic acid in the presence of histidine, since it employed a colour reaction characteristic of glyoxalines generally.

To determine whether histidine is at least partially converted to urocanic acid in the liver and subsequently broken down to glutamic acid by urocanase, it was essential to have available methods for identifying this possible intermediate in small quantities in the presence of histidine. The present paper records the use of two such methods. By these means, although no urocanic acid has been identified in whole liver extracts, presumably owing to the simultaneous presence of urocanase, an enzyme has been demonstrated which is capable of producing the unsaturated acid from histidine.

METHODS

Measurement of ultraviolet absorption

All the ultraviolet absorption data were obtained on a Beckman spectrophotometer, model DU. All the solutions were aqueous, and where values are given indicating differences in absorption between a test solution and a control, the absorption figures for each were measured separately against water as a blank and the difference obtained by subtraction.

Paper chromatography

Paper chromatograms of pure substances and of preparations from enzyme solutions were carried out in an upward

direction (Williams & Kirby, 1948) on Whatman no. 4 paper. Two solvent mixtures were employed: (a) butanol, 50 ml.; water, 50 ml.; glacial acetic acid, 12 ml., the alcoholic layer being used after equilibration; (b) a miscible mixture; butanol, 40 ml.; ethanol, 10 ml.; water, 50 ml.

The chromatograms were developed, after a run of 2-4 hr. at 30°, by spraying successively with a cold aqueous solution of diazotized *p*-chloroaniline (Edlbacher, Baur, Staehelin & Zeller, 1941) and *n*-NaOH. The latter spray could be replaced by suspension of the paper in an atmosphere of NH₃ vapour. The colours which varied between yellow and red persisted strongly, assuming a more intense red colour with age.

Preparation of urocanic acid

Urocanic acid was prepared by decarboxylation of a sample of 4(5)-glyoxalinylmethylenemalononic acid, which was kindly supplied by Dr A. Neuberger, F.R.S. The method of decarboxylation was essentially that of Akabori, Ose & Kaneko (1940) employing boiling pyridine. Most of this reagent was removed by distillation under reduced pressure and impure urocanic acid precipitated on pouring the residue into ether. Persistent washing with ether was necessary to ensure the removal of all the pyridine; the acid was then recrystallized from water to give material with a m.p. (uncorr.) of 226-228°. The absorption curve for this material is given in Fig. 1. Purity of the sample was determined in two ways. Freedom from pyridine was proved by the absence of the characteristic absorption peak of pyridine at 256 mμ. Freedom from malonic acid derivative was proved by partition chromatography. The *R_F* values of 4(5)-glyoxalinylmethylenemalononic acid and urocanic acid in butanol-acetic acid are respectively 0.30 and 0.52; thus separation is complete and small amounts of the malonic acid derivative can be detected in the presence of large quantities of urocanic acid.

A further sample of urocanic acid was obtained by an adaptation of the method of Engeland & Kutscher (1913) for the production of histidine betaine, and its subsequent breakdown to urocanic acid and trimethylamine by the method of Barger & Ewins (1911). The material prepared by this method had properties identical with those of the sample prepared from 4(5)-glyoxalinylmethylenemalononic acid.

Identification and estimation of urocanic acid

Histidine at pH 7.4 has a relatively low absorption peak at 262 mμ., and the molecular extinction coefficient of urocanic acid at the same pH is 200 times as great. Thus it is possible to use ultraviolet absorption as a method for the identification of urocanic acid in the presence of histidine.

The variation in intensity of absorption and in the position of the peak with changes in pH are such as could be expected with an amphoteric substance. The four curves in Fig. 1 demonstrate the magnitude of these pH variations. The curve at pH 4.5 represents the absorption of urocanic acid in aqueous solutions at a concentration of 4 μg./ml. The other curves are those for the same concentration of the acid brought to the stated pH by the addition of NaOH or HCl. It was found that in the presence of acid there was much irrelevant absorption with the systems under investigation and pH values around neutrality were, therefore, always employed when examining solutions for the presence

of urocanic acid. For although the peak at 278 $m\mu$. is lower it is well defined owing to the pronounced minima at nearby wavelengths.

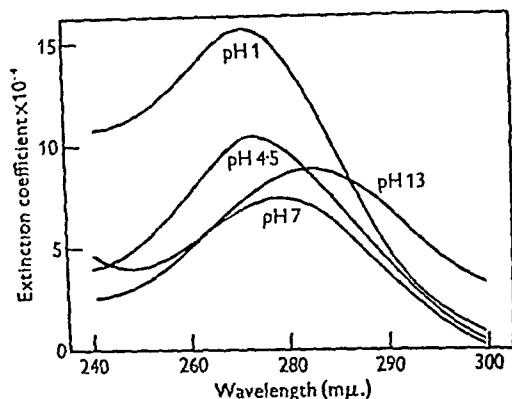


Fig. 1. Absorption curves of urocanic acid ($4 \mu\text{g./ml.}$), brought to the pH values indicated by the addition of appropriate amounts of sodium hydroxide and hydrochloric acid. The curve of pH 4.5 represents the actual curve of the acid dissolved in distilled water.

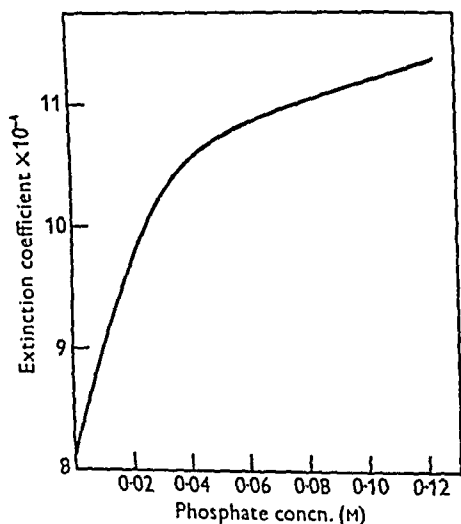


Fig. 2. The effect of phosphate ion concentration on the extinction coefficient at $\lambda_{\text{max.}}$ (278 $m\mu$.) for solutions of urocanic acid brought to pH 7 with sodium hydroxide.

Since in many of the systems to be examined, phosphate was present, its effect on the absorption was examined. As can be seen from Fig. 2, the absorption is considerably increased by the presence of even small concentrations of phosphate. Although the rate of increase falls off above a phosphate concentration of more than 0.05 M , there is a slight but steady increase at greater concentrations. The graph shows the absorption at 278 $m\mu$., and pH 7.6. This effect was also observed with borate; but not with other commonly occurring ions.

Solutions of urocanic acid, kept at normal temperatures in diffuse daylight, show variable changes in absorption. The value may fall by as much as 20% in 3-4 days, but the

presence of phosphate appears to retard the diminution of absorption.

Owing to the variations in absorption which are mentioned in the two preceding sections, it is impossible to obtain quantitative comparisons between solutions with entirely different pre-treatment, but for comparison of two solutions which have been treated in an identical manner, the method is regarded as quantitative. Over the range of 1-9 $\mu\text{g./ml.}$ in aqueous solution, the relationship between absorption and concentration of urocanic acid is linear. This covers the whole effective range of the spectrophotometer, using a 10 mm. cell. Higher concentrations can be read after dilution.

Examination of liver extracts

Choice and preparation of material. In most of the work reported here, cat-liver extracts were employed, but the technique was identical with extracts prepared from the livers of other animals. Livers were removed from cats killed by severance of the carotid artery and were washed free of superficial blood. The tissue was macerated to a fine cream in 2.5 times its weight of physiological saline. The grosser debris after 20 min. in the macerator (Townson & Mercer top-drive type) was removed by filtration after addition of kieselguhr equivalent to half the wet weight of the liver. The resultant opalescent solution was poured into 2 vol. of acetone at a temperature of less than 5°. The precipitate was filtered off at the pump. The cake, washed once with acetone, was dried at room temperature and stored at 0°. The powder was not completely soluble in water or the buffer used, but by agitation could be brought into a sufficiently fine suspension to withstand centrifuging under a force of 3500 g .

Properties of the extract powder. A sample of the original macerate, filtered through a 3 mm. layer of paper pulp on a sintered funnel, gave an indeterminate curve showing marked absorption in the range 240-300 $m\mu$., but no peak at 278 $m\mu$.. After ultrafiltration through a collodion membrane most of the absorption disappeared, showing it to be due to material of large mol.wt. There was no peak in the region of that associated with urocanic acid. Similar experiments on an aqueous or phosphate buffer extract of the acetone-dried powder prepared from the macerate failed to demonstrate the presence of any material with properties similar to urocanic acid. A sample of the extract from an acetone-dried powder that had been kept for 2 months at 0°, showed a marked peak in the neighbourhood of 278 $m\mu$.. Dialysis of the sample against running water for 24 hr. completely removed the peak, showing the material responsible for the absorption to be water-soluble and of low mol.wt.

Activity of liver powder

The incubation of a fresh acetone-dried liver extract with histidine caused a marked increase in absorption with a peak at 278 $m\mu$.. Such an increase was produced by a heat-labile system, no increased absorption being observed when histidine was incubated with boiled preparations. The same histidine α -deaminase is suggested for the enzyme concerned.

Method of testing enzyme activity. Except when various concentrations of enzyme and substrate were examined for the specific purpose of determining concentration effects,

the following procedure was employed for assessing the amount of enzyme in a given preparation. Enzyme powder (50 mg.) and solid phosphate buffer mixture, pH 7.6, (50 mg.) were dissolved in 5 ml. of water. To this mixture was added 5 ml. of a solution of histidine hydrochloride in water (1 mg./ml.) brought to pH 7.6 with NaOH. Water (5 ml.) was added in the case of the blank. The preparations were incubated at 30° for 18 hr. in the presence of 0.2 ml. of toluene in 50 ml. flasks covered with a 30 ml. beaker. After incubation the flasks were placed in boiling water for 5 min. to inactivate the enzyme, coagulate the protein and remove the small quantity of toluene. The solid was then removed by centrifuging, and the volume of the supernatant made up to 10 ml. 1 ml. of this solution was diluted with water to 50 ml., to give the solution for measurement in the spectrophotometer. The curves observed on incubation of the enzyme in the presence and absence of histidine are given in Fig. 3.

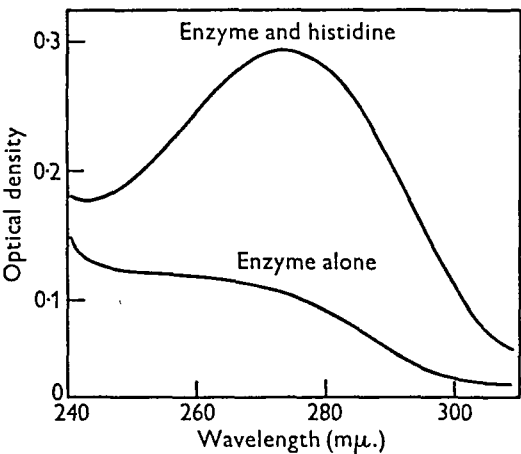


Fig. 3. Absorption curves of enzyme and enzyme-substrate mixtures after incubation at pH 7.8 for 18 hr. 50 mg. liver powder, 50 mg. phosphate buffer powder, and either 10 ml. of a 0.1% solution of histidine hydrochloride or 10 ml. of water.

Evidence for the identification of the enzyme product as urocanic acid

Examination of the material produced during the incubation of the enzyme preparation with histidine showed that changes in absorption brought about by pH were identical with those observed in pure solutions of urocanic acid. For this experiment buffer solutions or acid or alkali were used instead of water for the 50-fold dilution prior to measurement of the absorption. The changes observed are exactly as those shown in Fig. 1.

For the chromatographic identification of the material as urocanic acid all the quantities employed in the normal test were multiplied ten times. After the completion of the incubation period and after the coagulation and removal of most of the protein by filtration, the solution was concentrated to 3 ml. under reduced pressure, and the smaller molecules separated from the residual protein by ultrafiltration through a collodion membrane.

A chromatogram of this material in butanol/acetic acid showed two main spots and two fainter ones. The R_F values of these and of a number of other imidazole derivatives are given in Table 1. In butanol/ethanol/water the movement of the lower two spots was only slight, and only after very long runs did separation occur. The two main spots coincide with those for histidine and urocanic acid; the other two have not yet been identified.

Table 1. R_F values of certain glyoxaline derivatives in butanol/acetic acid/water and butanol/ethanol/water mixtures

(The two unknown substances 1 and 2, were produced by the action of the liver preparation on histidine.)

Substance	R_F	
	Butanol/acetic acid/water	Butanol/ethanol/water
Urocanic acid	0.50	0.43
Histidine	0.11	0.10
β -Glyoxalinypropionyl chloride	0.25	0.37
Glyoxalinylmethylene-malonic acid	0.30	—
Methylglyoxaline	0.39	0.52
Dicarboxyglyoxaline	0.0	0.09
Unknown substance 1	0.29	0.35
Unknown substance 2	0.13	0.14

Distribution of properties of the enzyme

The enzyme has been shown to be present in the livers of cats, rats, rabbits and dogs, but not in the following tissues: the pancreas, kidney, duodenum, stomach, spleen and thyroid of the pig or heart of the horse. Nor is it present in *Escherichia coli* cells, although a casual contaminant of a histidine solution, which proved to be a Gram-negative diplococcus, produced a filtrate containing urocanic acid after 3 weeks at 18°.

Table 2. The effect of changes in concentration of enzyme and substrate on the ultraviolet absorption of histidine/histidine α -deaminase systems, after incubation at 30° for 18 hr. at pH 7.6

(The difference in E_{max} at 278 m μ . was calculated by subtracting the curve obtained from the control enzyme preparation incubated without histidine, from that obtained after incubation in the presence of histidine. This also applies to the optical density figures in Table 3.)

Concentration of liver powder added (mg./ml.)	Difference in E_{max} . Concentration of added histidine (mg./ml.)			
	0.1	0.2	0.5	1.0
0.5	0.007	0.010	0.018	0.028
1.0	0.009	0.013	0.023	0.039
2.0	0.019	0.026	0.038	0.056
5.0	0.027	0.051	0.084	0.114

In Table 2 are shown the results of a typical experiment in which both the concentrations of the

enzyme and of the substrate have been altered. The constants of the enzyme reaction have not yet been determined, since they will have little significance until purer preparations are obtained.

Enzyme activity was completely suppressed at pH values below 5; the pH optimum was found to be in the neighbourhood of 7.8. Citrate and phosphate buffers were employed to cover the whole pH range, and the figures for those experiments with phosphate were corrected for the enhancing effect of that ion on absorption (Table 3).

Table 3. The effect of changes in pH on the ultraviolet absorption at 278 m μ . of histidine/histidine α -deaminase systems incubated for 18 hr. at 30°. The figures for the experiments with phosphate buffer are corrected for the enhancing effect of the phosphate ion (see Fig. 2)

Buffer	pH	Differences in optical density
Citrate	3.0	0.012
	3.9	0.016
	5.1	0.003
	6.1	0.019
Phosphate	7.0	0.012
	7.2	0.008
	7.4	0.019
	7.6	0.098
	7.8	0.166
	8.0	0.144
	8.2	0.092

Old enzyme preparations which show the marked increase in urocanic acid content mentioned above are almost inactive, measured by increase of absorption on incubation with histidine. However, after dialysis and the removal of the urocanic acid the activity of the system increases to its normal value (Fig. 4).

DISCUSSION

Since it contains three conjugated double bonds, urocanic acid is expected to show considerable absorption in the ultraviolet. In fact the increase over histidine with only two double bonds is about 200-fold. This raises the molecular extinction coefficient to a level at which it is of use in the estimation of the acid in biological materials. The variability of the absorption renders the method not fully quantitative, but it is still of use in comparing similarly treated solutions. When associated with partition chromatography, ultraviolet absorption gives an admirable method of identifying the acid with a reasonable degree of certainty, but awaiting chemical characterization for absolute proof.

The reason for the decay of absorption on standing may be associated with the possibility of *trans-cis* interconversion which Edlbacher & Heitz (1943) have shown to occur quite rapidly at 100° and to be

photocatalysed. Whether phosphate acts as a stabilizer of the *trans* form or enhances the absorption in some other fashion cannot yet be decided.

The absence of urocanic acid from fresh liver extracts is consistent with urocanic acid being on the pathway of histidine metabolism. Assuming that urocanase catalyses a faster reaction in the destruction of urocanic acid than histidine deaminase does in its production, not much urocanic acid would be expected to accumulate. The few

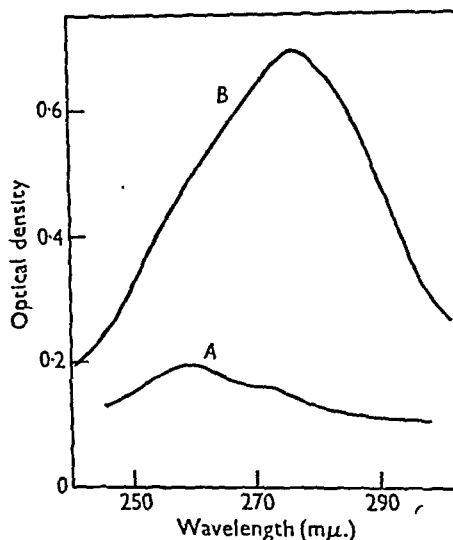
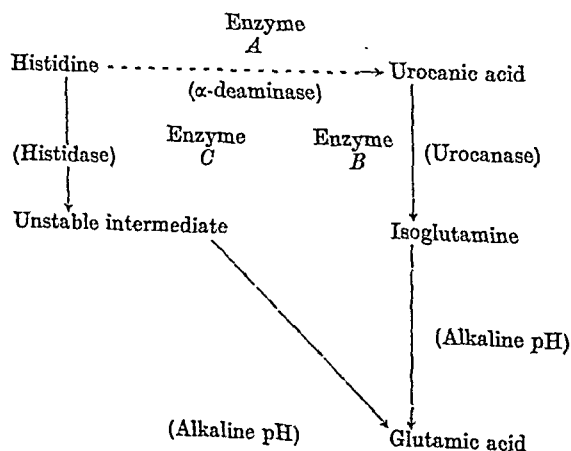


Fig. 4. Effect of dialysis on aged preparations. A, difference in optical density between enzyme and enzyme-substrate mixture of aged preparation before dialysis; B, similar curve obtained after dialysis of enzyme powder for 2 days against running water. The conditions of the experiments were in each case as given in legend to Fig. 3.

animals which have been shown to produce urocanic acid may not, therefore, have called into play a fresh metabolic pathway, but may merely have suffered the blocking of a single step in an enzymic chain. Until it is possible to measure quantitatively



the amount of urocanic acid produced it will be impossible to assess what fraction of the histidine is metabolized by the route involving urocanic acid.

The hypothetical system, shown on the previous page, proposed by Edlbacher would, however, appear to have more foundation in fact than he thought, since both enzymes *A* and *B* as well as *C* have now been shown to exist side by side in liver.

SUMMARY

1. Methods for the identification of urocanic acid (β -4(5)-glyoxalinyllacrylic acid) based on its ultra-violet absorption spectrum and on its separation

from other iminazole derivatives by partition chromatography are described.

2. No urocanic acid has been observed in fresh liver homogenates or extracts from acetone-dried powders obtained from aqueous extracts of liver.

3. An enzyme capable of producing urocanic acid from histidine has, however, been shown to be present in such aqueous extracts.

4. The name histidine α -deaminase is suggested for this enzyme.

5. The enzyme has an optimum pH in the region of 7.8.

6. It appears to be completely inhibited by the product of its reaction.

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Volatile Fatty Acids of *Ascaris lumbricoides* from the Pig

BY VIVIEN MOYLE AND E. BALDWIN*
The Biochemical Laboratory, University of Cambridge

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During a series of investigations of the pharmacology and physiology of *Ascaris lumbricoides*, extending from 1940 to 1949, our attention was constantly drawn to the peculiar and characteristic odour emitted by these parasites and by saline media in which specimens had been kept. Bunge (1890) appears to have been the first to attribute this odour to lower, steam-volatile fatty acids, but it was left to Weinland (1901, 1904) to show that the principal acids present are a valeric and a caproic acid. Similar substances are present in another parasitic nematode, *Parascaris equorum* (Schimmelpfennig, 1903). Flury (1912), working with *A. lumbricoides*, demonstrated the presence of volatile fatty acids, especially valeric and caproic, in the tissues of this worm as well as in saline media in which it had been kept. There has, in the past, been a good deal of discussion regarding the origin of these acids, some

attributing them to bacterial activity, while others maintain that they are produced by the worms themselves. The presence of relatively very high concentrations of these compounds in the body fluid and throughout the tissues would make it appear certain that the acids are produced by the worms themselves. A comprehensive review of earlier work on these acids has been given by Hobson (1948).

Although a dozen or more authors have studied the chemistry of these substances, their precise nature is still not definitely known. The results of Kruger (1936) were perhaps the most conclusive data published when our own work began, but even these are unconvincing. He collected the acids by steam-distillation of large volumes of saline in which his specimens had been accommodated. By fractional distillation of the product he obtained two main fractions, one consisting of a valeric and the other of a caproic acid, from both of which he prepared the anilides. He concluded that both acids

* Present address: University College, London.

are branched-chain compounds and, in the case of the valeric fraction (m.p. of anilide 98°), that it was neither the *n*-acid (m.p. of anilide 63°) nor the *iso*-acid (m.p. of anilide 112°), but must probably be the optically active methylethylacetic acid (m.p. of anilide of the racemic acid, 108°). Trimethylacetic acid was excluded on the grounds that it is solid at room temperature, while Kruger's fraction was liquid. The only confirmation in support of his identification of the optically active acid was the fact that mixtures of the anilides of the natural product and synthetic racemic acid, melted between 98 and 108°.

As we were able to obtain considerable amounts of the acids in the course of other investigations, we felt it was worth while to attempt a more positive identification. The work reported here was completed in 1949, but remained unpublished because we hoped at that time to carry it further. But in view of the recent work of Bueding & Yale (1951), it has seemed to us proper to record our observations. A brief account of this work was communicated to the Biochemical Society in London on 24 March 1950 (Baldwin & Moyle, 1950).

MATERIAL AND METHODS

Worms were obtained from the St Edmundsbury Co-operative Bacon Factory, to the manager of which we wish to express our gratitude for a regular and abundant supply of material. After washing in warm water, the specimens were conveyed to Cambridge in large thermos vessels containing the 'keeping medium' described elsewhere (Baldwin & Moyle, 1947), previously heated to 38°. On arrival in the laboratory the worms were drained, dried on filter paper, opened by a longitudinal incision and allowed to drain on a funnel, the stem of which was loosely plugged with glass wool. The clear, deep-red perienteric fluid thus collected, accounted for approximately one-third of the live weight of the worms and contained on the average about 40 m-moles of steam-volatile fatty acids per litre.

Proteins were precipitated by the addition of 0.8 vol. of 10*N*-H₂SO₄ and 1.0 vol. of 30% Na tungstate. The precipitate was removed in some cases by filtration and in others by

centrifugation. To each 100 ml. of the clear, colourless fluid 25 g. MgSO₄·7H₂O were added and allowed to dissolve. The whole was then distilled until crystallization began in the hot. The distillates were titrated with *N*-NaOH (phenolphthalein); the average yield was equivalent to 38.0 ml. *N*-NaOH/l. of perienteric fluid. A 10% excess of *N*-NaOH was added before evaporating the distillates *in vacuo* to about 2 ml. An excess of finely powdered KHSO₄ and two drops of 10*N*-H₂SO₄ were added, and the resulting dry powder was extracted in each case with about 50 ml. of CHCl₃ containing 5% (v/v) *n*-butanol in small successive portions.

Attempts were at first made to analyse the mixed acids by the distillation method of Friedemann (1938), and by fractional crystallization of the phenylhydrazides and piperazinium salts, before and after fractional distillation. The results obtained were discouraging, and we next tried the method of partition chromatography of Elsdon (1946). This method, however, did not allow separation between caproic and valeric acids or between these and butyric acid. We were accordingly led to investigate the possibility of using heavily buffered silica partition columns. Details for their preparation and operation have already been described (Moyle, Baldwin & Scarisbrick, 1948), and in particular the performance of columns I-III. In Table 1

Table 1. *Behaviour of aliphatic acids (C₂-C₆) on column IV*

Butanol (%)	Eluted	By-passed	Retained
1	C ₆	C ₆ (part) and higher	C ₂ and lower
10	C ₄		
30	C ₃		

characteristics of column IV, which uses as 'buffer' KH₂PO₄ only, are given. This method was applied to our present problem, after preliminary distillation of the aqueous solution in presence of HgO (Friedemann, 1938) to remove formic acid. The results obtained are summarized in Table 2.

RESULTS

Fraction C₁. It was found in preliminary experiments that the mixed acids, in the form of their sodium salts, decolorized iodine and bromine water, indicating the presence of small quantities of un-

Table 2. *Steam-volatile acids obtained from A. lumbricoides*

(F₁ and F₂ were duplicate analyses carried out on one sample.)

Sample	Percentage of total steam-volatile acids					
	C ₈	C ₆	C ₅	C ₄	C ₃	C ₂
B	1.48	40.9	40.0	1.14	4.06	12.4
C	0.78	41.2	43.5	1.72	4.73	8.23
D	29.8		50.8	2.75	4.31	12.6
E	0.37	34.1	46.2	1.89	5.12	12.6
F ₁	33.8		49.4	—	3.78	—
F ₂	33.7		49.3	—	3.93	—
Averages	35.7		46.0	1.9	4.4	11.1

saturated or reducing substances. Preliminary distillation of the acids in the presence of HgO , as recommended by Friedemann (1938) for removal of formic acid, yielded distillates which failed to react with either iodine or bromine water. It seems possible that the reducing material consisted of formic acid, small amounts of which have been found in *Ascaris* by other workers, e.g. Flury (1912). The amount present, calculated on the basis of iodine value, corresponded to only about 2% of the total acids present, and we therefore did not concern ourselves further with this fraction.

Fractions C_2 and C_3 . C_2 and C_3 together accounted for 15.5% of the total acids and corresponded in their chromatographic behaviour to acetic (11.1%) and propionic (4.4%) acids respectively.

Fraction C_4 . To obtain enough of the C_4 acid for identification we used silica partition columns 30 mm. in diameter, each containing 20 g. of silica and proportionate quantities of the other reagents. The original CHCl_3 -butanol extract (80 ml.) was placed on a column of type II and the large C_6 and C_5 fractions eluted, the first with 1% butanol in CHCl_3 , and the second with 10% butanol in CHCl_3 . The column was then allowed to run dry, the silica removed, and, after intimate admixture with an excess of finely powdered KHSO_4 , extracted with 100 ml. of 10% butanol in CHCl_3 , in successive portions. The extract was concentrated by adding a small excess of N-NaOH , evaporating to dryness, acidifying in the usual way and extracting with 20 ml. of 5% butanol in CHCl_3 . The whole extract was rechromatographed on a type IV column of standard size (5 g. silica) and the C_4 fraction eluted. A second 80 ml. sample of the original CHCl_3 -butanol extract was worked up in the same way. The combined C_4 fractions were now neutralized, recovered and again chromatographed on a type IV column. Titrations were carried out in the usual manner until the C_4 acid began to be eluted and subsequent samples were collected without titration until the whole fraction had been eluted. Titration of a small sample indicated that, in all, 19 mg. of the C_4 acid had now been obtained in 60 ml. of extract. The theoretical amount of piperazine hexahydrate in ethanol was now added and the whole evaporated under reduced pressure. On addition of a few drops of acetone to the resulting oil immediate crystallization took place. After standing overnight at 3° the product was filtered off (m.p. 115–117°; mixed m.p. with the piperazinium salt of *n*-butyric acid 116–117°). The product was recrystallized from acetone (m.p. 117–118.5°; mixed m.p. with the derivative of isobutyric acid 65° approx.).

The C_4 acid is thus identified as *n*-butyric acid. (All m.p.'s uncorr.).

Fractions C_5 and C_6 . Samples of the mixed acids were put on to 20 g. columns of type II. The C_6 fraction was eluted with 1% butanol in chloroform and the C_5 acids with 10% butanol in chloroform. Fractions obtained in this way from each of several columns were massed together and purified on 20 g. columns of type III, from which the C_6 acids were eluted with 10% butanol in chloroform, and C_5 acids with 30% butanol in chloroform.

After recovery of the acids in the usual way, the fractions were separately treated with the theoretical amounts of piperazine hexahydrate, in repeated attempts to obtain crystalline derivatives, but in every case oily products were obtained which failed to crystallize, even on prolonged standing. Since the piperazinium derivatives of all four isomers of valeric acid and of *n*- and *iso*-caproic (hexanoic) acid were readily obtained individually from pure samples of each, we concluded that the natural C_5 and C_6 fractions consisted of mixtures containing more than one isomer in each case. Further chromatographic experiments were therefore made in the hope of resolving the mixtures.

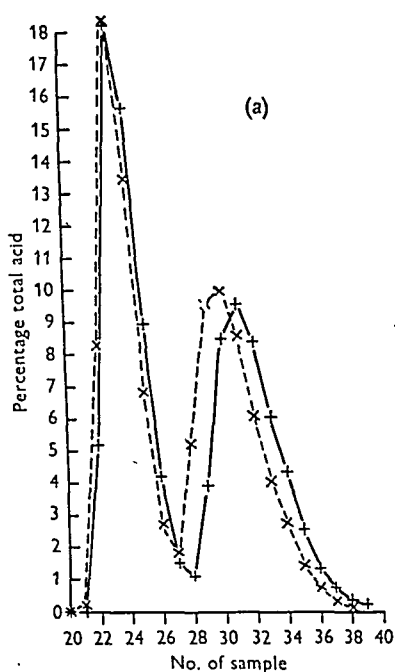
Chromatographic examination of C_5 fraction

Partition analysis. The behaviour of the C_5 fraction on our usual buffered columns (type III) is depicted in Fig. 1, which also shows the results of running a series of mixed chromatograms with known acids. As previously shown (Moyle *et al.* 1948), *n*-valeric and trimethylacetic acids are separable from isovaleric and methylethylacetic acids, but neither pair can be resolved by this method. The C_5 fraction from *A. lumbricoides* behaves in the same way as isovaleric and methylethylacetic acids, whether separately or in mixtures, and comes through significantly later than *n*-valeric and trimethylacetic acids. This led us to the conclusion that the C_5 fraction must include one or more of three compounds, namely, isovaleric, (+)- and (–)-methylethylacetic acids.

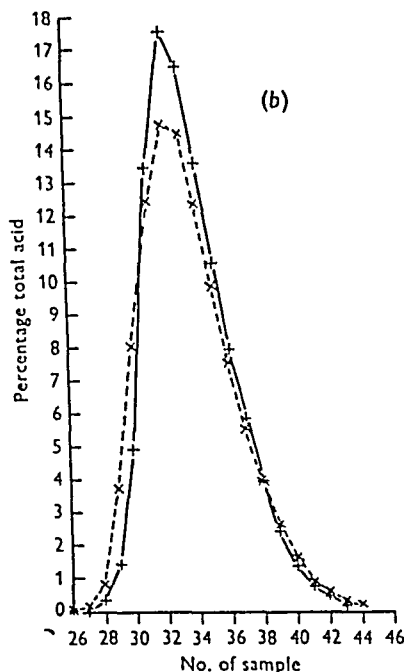
Adsorption analysis. Following the indications given by Claesson (1946), we carried out experiments on short adsorption columns of silica, previously dried at 800°, using pure specimens of the valeric acid isomers dissolved in cyclohexane. In this way it was found that *n*-valeric and trimethylacetic acids could be separately estimated by frontal analysis; mixtures of either, with other isomers or with the worm acid(s), could not be analysed. Mixtures of isovaleric and methylethylacetic acids showed no signs of separation.

Although this part of our work failed to carry us any further, it is mentioned here since *n*-valeric and trimethylacetic acids are inseparable by our partition columns. Their separation and estimation by frontal analysis on adsorption columns thus forms a useful supplement to the partition technique.

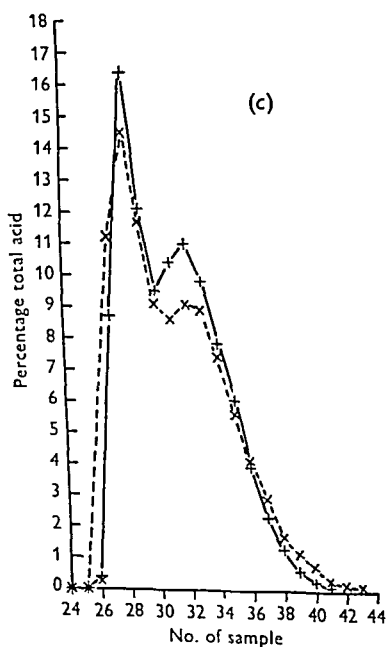
Further examination of C_5 and C_6 fraction. Having failed so far in our attempts to resolve either the C_5 or C_6 fractions into their isomeric components by chromatographic methods, we now reverted to more classical techniques involving the use of larger quantities of material. The acids used in this part of the work were obtained by steam distillation of large, bulk samples of intact worms previously submitted to digestion by 30% (w/v) sodium



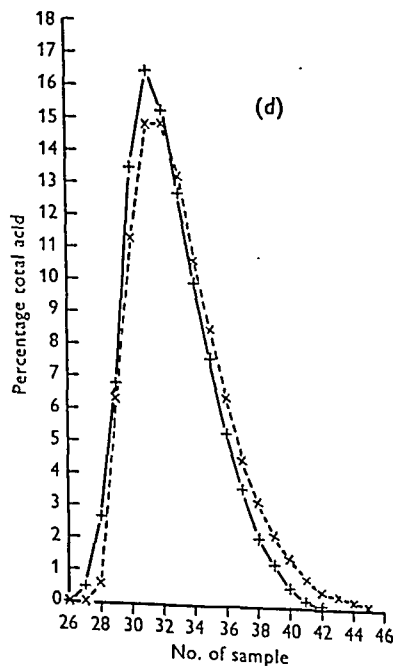
×---×, Trimethylacetic + methylethylacetic acid;
+—+, trimethylacetic + isovaleric acid.



×---×, Worm acid; +—+, worm + methylethylacetic acid.



×---×, iso- + n-Valeric acid; +—+, worm + n-valeric acid.



×---×, isoValeric acid; +—+, worm + isovaleric acid.

Fig. 1. Chromatographic behaviour of C_5 worm acid and isomers of valeric acid; mixed chromatograms.

hydroxide, first for several weeks at room temperature and then on the boiling-water bath, and subsequently acidified with sulphuric acid.

Approximately 9 g. of the mixed worm acids were refluxed for 5 hr. with 40 ml. dry methanol containing 0.5 ml. conc. H_2SO_4 . Methanol (30 ml.) was removed by distillation and the residual mixture of methyl esters extracted with ether. The ethereal layer was washed five times with 2% aqueous K_2CO_3 , dried over K_2CO_3 and distilled to remove ether. The mixed esters were now fractionally distilled from a small flask of about 25 ml. capacity. The following fractions were collected:

Fraction	B.p. (°)	Weight (g.)	Probable composition
1	34–36	—	Ether
2	36–100	0.86	Methyl esters of acetic, propionic and butyric acids
3	100–120	2.02	Methyl esters of valeric acids
4	120–132	2.43	Methyl esters of caproic acids

Since fraction 4 contained two main sub-fractions, boiling at 129° and 131–132°, it seems probable that two isomeric forms of caproic acid are present. These cannot be either *n*-caproic (b.p. of methyl ester 150°) or methylethylpropionic acid (b.p. of methyl ester 141°) and must therefore be other branched-chain caproic acids. The fraction was found to be optically active ($[\alpha]_D = -2.245^\circ$) and must therefore contain at least one optically active acid.

The main part of fraction 3 came over at 115–117° and might consist of the methyl esters of *isovaleric* (b.p. 116°) and (+)- and (–)-methylethylacetic acids (b.p. 113–115°). This fraction, too, proved to be optically active ($[\alpha]_D = -1.405^\circ$) and must, therefore, contain at least some methylethylacetic acid. Because their boiling points lie so close together, there seemed little prospect of analysing this mixture of esters by fractional distillation, and still less of analysing the corresponding mixture of acids ((±)-methylethylacetic acid, b.p. 173°; *isovaleric* acid, b.p. 174°). As our supplies of the acids were now running low we made no further attempts to fractionate the C_5 acids by distillation methods, but turned instead to infrared spectroscopy. The C_5 fraction of the worm acids (1–2 g.) was obtained in the pure state by repeated chromatography as for the C_4 fraction, described above. The acids were titrated with *N*-sodium hydroxide, the sodium salts thus obtained distilled to dryness, and dried *in vacuo* over phosphorus pentoxide. A pure, dry sample of the free acids was obtained by intimately mixing the dry sodium salts with finely powdered dry potassium hydrogen sulphate and distilling the acids in a flask similar to that used by Ellis (1934).

In order to determine whether the C_5 acid obtained above was methylethylacetic acid or *isovaleric* acid, the infrared spectra of pure samples of these acids and of the unknown were determined. The spectrometer used was a Perkin Elmer Type 12B, and the spectra were obtained between 1800 and 700 cm^{-1} using a rocksalt prism. The liquid samples were approximately 0.005 mm. in thickness. The absorption curves obtained are shown in Fig. 2.

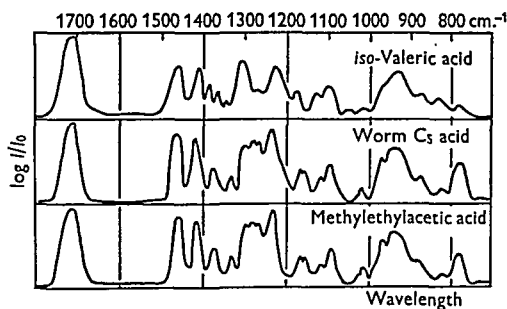


Fig. 2. Infrared absorption spectra of C_5 acids.

Although the two pure acids have a number of absorptions in common, there are considerable differences in their spectra, notably in the region 1550–1150 cm^{-1} . In particular, *isovaleric* acid has strong absorption peaks close to 1300 and 1220 cm^{-1} , whereas methylethylacetic acid has a marked absorption distinct from those of *isovaleric* at about 1230 cm^{-1} . It can be seen immediately from the figure that the spectrum of the unknown material very strongly resembles that of methylethylacetic acid throughout the region investigated, and differs in many respects from that of *isovaleric* acid. The only detailed difference between the spectrum of the unknown acid and of pure methylethylacetic acid is an additional faint absorption in the former, which shows as a shoulder in the spectrum near 1210 cm^{-1} . This presumably corresponds to a small amount of impurity, which might possibly consist of a little *isovaleric* acid. It is clear, however, that the unknown acid is very nearly pure methylethylacetic acid, with an impurity content of probably less than 10%.

It is not possible, of course, to distinguish by spectroscopic means between the (+)- and (–)-forms of this acid. The polarimetric data already mentioned indicate that, in the acid as analysed, there was a small preponderance (about 6%) of the (–)-isomer. There remained, however, the possibility that extensive racemization might have taken place in the course of preparation of these particular batches of acids. The most likely step at which racemization might have occurred was during the digestion of the worms with 30% sodium hydroxide for some weeks in the cold, followed by several hours in the hot. This possibility was investigated in the following manner.

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Fraction C_8 . This fraction consisted of a mixture of acids containing more than six carbon atoms, and, since it accounted for only 1% of the total acids, was not examined further.

DISCUSSION

The presence of formic, acetic, propionic and *n*-butyric acids in a wide range of biological materials is well known, and they are, moreover, among the principal products of the digestion of cellulose by the rumen contents of the sheep and other herbivores (McAnally & Phillipson, 1944). That these acids may also be formed by bacterial activity in the alimentary tract of the pig is possible, and the fact that they occur in laboratory media in which specimens of *Ascaris* have been incubated has led many workers to the conclusion that they are bacterial in origin. But these acids are present in relatively high concentrations in the perienteric fluid of freshly collected worms, suggesting that they escape into the keeping medium by diffusion or, perhaps, by excretion. However, these lower acids together account for less than 20% of the total volatile acids present in the perienteric fluid. The residual methylethylacetic acid (46%) is optically active, and the C_6 acid (35%) also appears to be optically active. Optically active isomers of valeric and caproic acids are not known as products of microbial digestion, at any rate of cellulose, nor, so far as we are aware, have they heretofore been dis-

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Percentage of total steam-volatile acids					
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36	46	2	4	11	Present work
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But these differences are less important than those between our respective conclusions regarding the nature of the C_6 acid; for whereas we believe it to consist almost in its entirety of methylethylacetic acid, Bueding & Yale (1951) state that about one-half consists of *n*-valeric acid and the other half of a mixture of isovaleric and methylethylacetic acids. Small amounts of an $\alpha\beta$ -unsaturated C_6 acid were also reported. We ourselves found traces of a substance which decolorizes bromine water and which might be formic or some unsaturated acid. It is difficult to reconcile our respective conclusions or to explain the differences that exist; but it is conceivable, since much of the C_6 acid has apparently been transferred to the lower fractions in Bueding & Yale's experiments, that these workers were, in fact, dealing with partial degradation products formed—most probably by some kind of microbial activity—from acids which had diffused out of the worms into the culture medium. They themselves deny that bacterial activity was involved in their experiments, on the grounds that the worms used were treated with antibiotic substances. But antibiotics in general are more or less specific in their action, so that these allegedly sterile experiments may reasonably be regarded with some suspicion.

The origin and mode of formation of these acids is wholly obscure. In the past they have often been compared with the lactic acid which can arise anaerobically from carbohydrate, e.g. in muscle and in a variety of bacteria, for it is generally supposed that *Ascaris* itself lives under conditions that approximate fairly nearly to anaerobiosis (see Hobson, 1948). However, the structure of methylethylacetic acid, which contains an asymmetrically branched carbon chain and accounts for practically one-half of the total acids, suggests that the latter can hardly arise from carbohydrate sources. The branched chain of methylethylacetic acid occurs,

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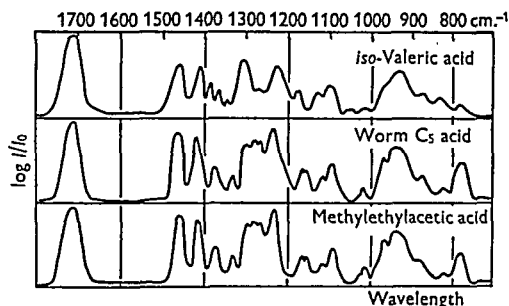


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20	40	2-5	10-15	15-25	Bueding & Yale (1951)

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however, in isoleucine; it is therefore conceivable that our C₅ acid might arise by oxidative deamination of isoleucine, followed by oxidative decarboxylation of the resulting α -keto acid. These are reactions that are known to occur among vertebrates and probably go on in invertebrates also. Perhaps they are less likely to take place in an animal that leads what appears to be a largely or mainly anaerobic existence, though even here oxidative processes might occur by coupling to simultaneous reductive reactions. But in any case, unless we assume that isoleucine alone is singled out for this kind of treatment we should expect leucine itself to give rise through similar reactions to *isovaleric* acid, and the latter cannot account for more than 10% of the total valeric fraction, according to the spectroscopic evidence. Valine, moreover, would be expected to give rise in a similar way to *isobutyric* acid, whereas our C₄ acid is the *n*-compound. Nor does such a mechanism allow us to explain in any simple manner the relatively large-scale formation of a C₆ acid.

Other mechanisms might be invoked to account for the formation of methylethylacetic acid from isoleucine, the only other commonly occurring biological compound known to contain the appropriate branched chain. We have considered several possibilities of this kind, all of which lead to conclusions that are inconsistent within themselves,

like the scheme discussed here. We must at present confess to complete ignorance of the origin, mode of formation and function of these curious compounds.

SUMMARY

1. The steam-volatile fatty acids of the perienteric fluid of *Ascaris lumbricoides* have been analysed by chromatographic methods.

2. Acetic, propionic and *n*-butyric acids have been identified by their chromatographic behaviour and by the piperazinium salt of *n*-butyric acid. Small quantities of formic acid also appear to be present.

3. Infrared spectroscopy showed that the C₅ fraction consists, almost in its entirety, of the optically active methylethylacetic acid (2-methylbutanoic acid). There is a slight preponderance of the laevorotatory form.

4. The C₆ fraction has not so far been identified.

5. It is concluded that these acids are products of the undoubtedly peculiar metabolism of *A. lumbricoides* and are not formed by microbial activity. Their origin, function and mode of formation are unknown.

We wish to thank Dr N. Sheppard for determining the infrared absorption spectra of the acids examined by this method.

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Cacao Polyphenolic Substances

1. FRACTIONATION OF THE FRESH BEAN

By W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Extremely little is known about the polyphenolic substances of cacao. Numerous analyses have been made but few isolations. To obtain a rational idea of the changes taking place during the fermentation process in the preparation of the bean for the market, the chemistry of the polyphenolic substances present must be placed on a sounder basis. This is only possible by the isolation of the different substances from the fresh bean.

A catechin was isolated by Adam, Hardy & Nierenstein (1931) and by Freudenberg, Cox & Braun (1932), who showed it to be (-)-epicatechin. It is claimed (Adam, 1928) that the epicatechin is present at least in part as a catechin-caffeine complex. Various crude 'tannin' preparations have been isolated (Adam, 1928; Mosimann, 1944). Other substances have been shown to be present by qualitative reactions. The purple colouring matter is due to anthocyanins (Laurence, Price, Robinson & Robinson, 1938) having the colour and solubility reactions of a cyanidin-3-glycoside. Leucoanthocyanins are also present (Knapp & Hearne, 1939).

Numerous proximate methods of determining the 'tannin' of cacao have been developed, few of which have led to any understanding of the substances present. The sum of these studies has shown, however, that the material is a complex mixture of fractions of different solubility. Extractions have been made with ethyl acetate, water (Adam, 1928), neutral buffer, sodium hydroxide, acetic acid (Hallas, 1939), 40% acetone (Duthie, 1938), and dilute hydrochloric acid (Hallas, 1949). The greatest amount determined is about 15% of the dried bean (Hallas, 1939). Of this about two-thirds can be extracted with aqueous acetone, alcohols, or dilute acids, and roughly comprises the fraction studied here. Hallas (1949) has shown that the anthocyanins and leucoanthocyanins in the fresh purple bean are nearly completely soluble in ethanol.

If any success is to be gained in the separation of what is probably a complex mixture of polymers of different degrees of condensation new methods are obviously required. Bradfield, Penney & Wright (1947) and Bradfield & Penney (1948) have had considerable success in separating the simpler polyphenolic substances in tea by partition chro-

matography on silica gel. White (1949) has shown that the complexity of quebracho tannin extract can be gauged by paper chromatography. Bate-Smith (1949) has demonstrated that anthocyanins and flavones and many similar substances are separable by paper chromatography. A preliminary note (Forsyth, 1949) has been published on the paper chromatography of extracts of cacao beans.

METHODS AND RESULTS

Paper chromatography

Twenty fresh beans were removed from the pod, peeled, and immediately dropped into 100 ml. HCl (0.3N). The acid suspension of cotyledons was homogenized in a Waring Blender for 3 min. After standing for 15 min. at room temperature (25°) the homogenate was filtered and the extract (20 μ l.) spotted on the chromatogram.

The most useful solvent for development was found to be amyl alcohol-acetic acid-water in the proportions 4:1:5. *n*-Butanol-acetic acid (Partridge, 1948) gave similar results, but with cacao extract the spots were not so well separated although they moved much faster. Butanol-acetic acid was used to confirm the identity of the separated substances under the rigid conditions specified by Bate-Smith & Westall (1950). The amyl alcohol-acetic acid chromatograms were run on Whatman no. 1 paper for 18 hr. at 25°. The substances were detected by visible colour and by spraying with ammoniacal AgNO₃. Aniline hydrogen oxalate (Partridge, 1949) was used to detect sugars.

Table 1. *Paper chromatogram of a 0.3N-HCl extract of fresh Forastero cacao beans*

(Solvent, amyl alcohol-acetic acid-water.)		
No. of spot	R _F	Probable nature
7	0.40	Epicatechin
6	0.22	Leucoanthocyanidin
5	0.16	Cyanidin-anthocyanin I
4	0.12	—
3	0.08	Cyanidin-anthocyanin II
2	0.04	—
1	0.002	Cyanidin diglycoside leucoanthocyanin

A typical chromatogram showing the main polyphenolic components of an HCl (0.3N) extract of fresh Forastero (purple) beans is shown in Table 1. These substances were invariably present in fresh Forastero beans from many different pods and were the major components.

By extracting the spots from large numbers of papers evidence has been produced (Forsyth, 1949), using Robinson

& Robinson's (1931, 1932) tests, that the components are as stated in the table. Isolation is necessary for better characterization.

As well as the two mobile anthocyanins there was also a trace of anthocyanin not extracted with amyl alcohol (spot no. 1) which had the properties of a cyanidin diglycoside. It hydrolysed to cyanidin and had the solubility of a diglycoside (R_F butanol-acetic 0.17).

Comparison of different beans. Twelve individual beans from the same Imperial College Selection 1 (I.C.S. 1) pod were extracted. They gave identical chromatograms. Twelve individual beans from twelve different I.C.S. 1 pods were extracted. They gave identical chromatograms. Beans of Criollo (non-pigmented) varieties give the same spots except that the anthocyanins are absent. The leucoanthocyanin was present in relatively greater quantity.

The following species and varieties used in chocolate manufacture have been examined and the same spots are present in all the purple beans and only the anthocyanin pigments are missing in the white beans: Red Venezuelan, Nicaragua Criollo, Ecuador Nacional, Trinitario, Calabacillo, *Theobroma pentagona*, *Th. leiocarpa* (Cheesman, 1944). The associated species *Th. bicolor* which is not used contained none of these compounds.

When white, purple-bordered, faint-purple, medium, strong and very strong purple beans were extracted the two anthocyanins were either both absent or both present in the same relative proportion.

Isolation

Imperial College Selection 1 pods were used. It was first necessary to determine what pretreatments of the beans to aid extraction were safe. It was found that fresh beans could be washed in an adapted clothes-washing machine to remove the pulp completely in 2 hr. and then could be heat-killed in boiling water for 5 min. and dried in the oven at 70° in 12 hr. without change in the acid-soluble compounds. In such heat-killed beans the enzymes were completely destroyed. The dried beans were readily peeled and the butter was partly removed by blending with three successive lots of light petroleum (90–100°, 1 ml./g.) at 60°. This procedure removed over 90% of the butter giving a readily extracted stable residue which on extraction gave a chromatogram qualitatively identical to that obtained from fresh beans.

The anthocyanins

Preparation of a concentrate. Attempts to concentrate the anthocyanins via the picrates were unsuccessful, even at concentrations where picric acid crystallized out the pigments remained in the mother liquors. The anthocyanins could, however, be readily concentrated through their lead salts. The method was basically that of Scott-Moncrieff (1930).

Wax-free cacao (100 g.) was blended with 500 ml. 0.3N-methanolic HCl and left overnight. The extract was filtered off and the process repeated. To the combined extract was added a saturated aqueous solution of lead acetate. The lead chloride which first precipitated was removed and then excess lead acetate added to bring down the pigments as a dark-blue precipitate. After standing overnight the precipitate was filtered off, washed with methanol, and dissolved in the minimum amount of glacial acetic acid (about 100 ml.). This extract was then treated with 2 vol. of ether.

The blue lead salt precipitate was washed with ether, dried and ground. The powder was extracted with 25 ml. methanol containing 5% (v/v) HCl and the crude glycosides thrown down as a bright-red gummy mass by addition of 12 vol. ether. The pigments were further purified by extraction with ethyl acetate.

The preliminary purification could also be done simply by ethyl acetate extraction. Wax-free cacao (100 g.) was extracted with 2 × 500 ml. 0.3N-methanolic HCl as before. The extract was diluted with 2 vol. ether and 4 vol. light petroleum and the crude anthocyanin precipitate was washed successively with light petroleum and ether. It was then extracted exhaustively with ethyl acetate to remove impurities soluble in ethyl acetate.

The simplest method of extraction is with methanol containing 10% acetic acid. After filtration the methanol is removed *in vacuo* and the anthocyanins precipitated by addition of a large excess of ether to the acetic acid. The solid is then washed with ethyl acetate.

Chromatography of the anthocyanin concentrate on columns

Chromatography on alumina, silica gel or kieselguhr did not give good results. The most satisfactory chromatograms were obtained on columns of cellulose pulp (Solka flock 200 mesh, Johnsen, Jorgensen and Wettre Ltd., London). The material, purified as described by Campbell, Work & Mellanby (1951), was packed dry in columns (25 × 3.7 cm.) and washed well first with the aqueous and then with the solvent layer of amyl alcohol-acetic acid.

The anthocyanin concentrate prepared by any of the above methods was taken up in the organic solvent layer of amyl alcohol-acetic acid and added to the column and developed with the same solvent (500 ml.). The column was sucked dry, extruded, and the two individual anthocyanin bands cut out separately and, after being allowed to dry at room temperature, were eluted with methanol containing 0.5% (w/v) HCl. Paper chromatograms of the extracts showed the anthocyanins uncontaminated with any substances reacting with Na_2CO_3 , FeCl_3 , or ammoniacal AgNO_3 .

The anthocyanins could be precipitated as bright-red gums by the addition of a very large excess of ether to the methanolic HCl extracts from the column. Attempts at crystallization of the small amount of either gum from methanol, ethanol or water containing various amounts of HCl have been unsuccessful. The anthocyanins can be precipitated from methanol solution with ether as dark-purple amorphous powders. These two powders are chromatographically homogeneous and both give cyanidin on hydrolysis with 20% (w/v) HCl, confirmed by paper chromatography in *n*-butanol-2N-HCl (Bate-Smith, 1949) R_F = 0.70 and by colour reactions (Robinson & Robinson, 1931). Anthocyanin I is present in about three times the concentration of anthocyanin II as judged by the intensity of the spots.

Anthocyanin I. The R_F value under the standard conditions of Bate-Smith (1949) in butanol-acetic acid is 0.41.

The amorphous powder (10 mg.) was hydrolysed for 30 min. in 0.5N- H_2SO_4 (0.5 ml.) at 100° in a sealed capsule. The hydrolysate was neutralized with BaCO_3 , centrifuged and concentrated *in vacuo*. The residue was extracted with methanol and the methanol concentrated to 0.2 ml. This solution gave positive reactions with benzidine and orcinol tests. Paper chromatography showed that two sugars were

present. Spraying with selective reagents (Forsyth, 1948; Partridge, 1948) showed the two spots to be an aldohexose and an aldopentose. The two sugars could not be separated from glucose and arabinose when chromatographed in butanol-acetic acid, phenol (Partridge, 1948), or ethyl acetate-pyridine (Jermyn & Isherwood, 1949).

The pentose was readily separated from added xylose, ribose and lyxose, and the aldohexose readily separated from added galactose and mannose. Quantitative determination of the proportions of the two sugars in the chromatograms (Flood, Hirst & Jones, 1947) showed them to be present in the ratio of 1:1.

That the anthocyanin should be a simple 'cyanidin-glucose-arabinose' with an R_F of 0.41 is very unlikely when compared with the known cyanidin anthocyanins (Table 3). It is most probably acylated. This was confirmed by a brief alkaline hydrolysis for 30 sec. (Robinson & Robinson, 1931). The acyl group was removed and the anthocyanin now had an R_F of 0.35 which is in closer agreement with expectations on theoretical grounds.

Anthocyanin II. The R_F value under standard conditions in butanol-acetic acid is 0.33. The amorphous powder (10 mg.) was hydrolysed with 2N- H_2SO_4 and treated as was anthocyanin I. Chromatography of the sugar solution showed only a single aldohexose to be present which was not separable from glucose in the three solvent mixtures, but was readily separated from galactose or mannose. The anthocyanin was unaffected by brief alkaline hydrolysis, i.e. it is a simple cyanidin monoglucoside.

Non-mobile fraction

On paper chromatography of an HCl or methanolic HCl extract with amyl alcohol-acetic acid much of the material remains on the starting line. This spot contains a trace of an anthocyanin, but also considerable material, which gives cyanidin on boiling with conc. HCl, and reacts with ammoniacal $AgNO_3$.

An anthocyanin concentrate was prepared from wax-free cacao (100 g.) by methanolic-HCl extraction and ether-light petroleum precipitation as previously described. On

extracting the anthocyanins from the concentrate with amyl alcohol-acetic acid a considerable residue was left. This residue was dissolved in 0.3N-methanolic HCl (250 ml.) and reprecipitated with ether (2 vol.) three times. The product was then washed with ether and dried to give a brick-red residue which on paper chromatography remained on the starting line.

On boiling with 20% (w/v) HCl a coloration due to liberated cyanidin (R_F in butanol-2N-HCl, 0.69) was produced. Hydrolysis with 2N- H_2SO_4 followed by neutralization with $BaCO_3$ gave a sugar solution, shown by chromatography to contain glucose and arabinose in the proportion of roughly 3:1.

The ethyl acetate fraction

Wax-free cacao (100 g.) was extracted with two successive litre lots of 0.3N-HCl. The filtered extract was shaken with three successive 500 ml. lots of ethyl acetate. The ethyl acetate was dried (Na_2SO_4), the solvent removed under reduced pressure (nitrogen bubbler) and the residue transferred in boiling $CHCl_3$ to a sintered-glass funnel, washed with $CHCl_3$ and dried. About 3 g. of a light-tan coloured amorphous powder were obtained containing the substances which give 'AgNO₃-positive' spots on the paper chromatograms. The extraction with $CHCl_3$ had no effect on the pattern of any of the spots, showing that by this method of extraction no catechin-caffeine complex was present.

The powder was taken up in a small volume of the top layer of the amyl alcohol-acetic acid-water mixture and transferred to a cellulose pulp column (12.5 × 3.7 cm.) and the column developed with the same solvent. The eluate was collected in 2 ml. lots and spotted on paper chromatograms. Suitable cuts were made and similar fractions combined. Each fraction was then taken to dryness *in vacuo* and the residue washed with $CHCl_3$. The results are shown in Table 2.

Cut A. Attempts to obtain any crystalline products from cut A were unsuccessful. On paper chromatography a series of fast-moving brown and yellow streaks were observed. No spot was detected on spraying with aqueous NaCN or vanillin-HCl (Bradfield *et al.* 1947), or with acetic acid-

Table 2. Fractionation of the ethyl-acetate solubles (3 g.) on a cellulose column with amyl alcohol-acetic acid

Cut	Vol. eluate (ml.)	Dry wt. (mg.)	Components provisionally identified	R_F		
				Amyl alcohol-acetic acid	Butanol-acetic acid	Phenol-water
O	40	—	—	—	—	—
A	34	90	o-Hydroxyphenols absent	0.75-0.95	—	—
B	48	1028	(-)-Epicatechin	0.40	0.65	0.48
			Catechin	0.50	0.74	0.36
C	26	304	(-)-Epicatechin	—	—	—
			Gallocatechin	0.27	0.57	0.25
D	78	578	(-)-Epicatechin	—	—	—
			Gallocatechin	—	—	—
			Epigallocatechin	0.22	0.48	0.32
			Leucoanthocyanidin	0.19	0.40	—
E	98	169	Leucoanthocyanidin	—	—	—
			Epigallocatechin	—	—	—
F	144	84	Spot no. 4 (Table 1)	0.12	0.44	0.21
			Spot no. 4 (Table 1)	—	—	—
			Trace of anthocyanin I	—	—	—
G	200	22	Spot no. 2 (Table 1)	0.04	0.39	0.12

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ammonium molybdate (Quastel, 1931), i.e. no catechins or other *o*-hydroxyphenols were present.

Cut B. Cut *B* consisted mainly of the major catechin (spot no. 7 in Table 1) with traces of another compound. Both substances were catechins since they could be detected by spraying with ammoniacal AgNO_3 , FeCl_3 , Na_2CO_3 , ammonium molybdate, NaCN and vanillin-HCl solutions.

The powder from cut *B* crystallized from water. The major catechin was the more insoluble and the material was recrystallized till the crystals were chromatographically homogeneous. In all 722 mg. of colourless short prisms were obtained, m.p. 237° (uncorr.); $[\alpha]_D^{20} - 58^\circ$ ($c=2$ in aqueous acetone, 1/1). (Found: C, 49.6; H, 6.2. Calc. for $\text{C}_{15}\text{H}_{14}\text{O}_6 \cdot 4\text{H}_2\text{O}$: C, 49.7; H, 6.1.) The acetyl derivative was prepared, m.p. 151° .

The pure crystalline material was responsible for spot no. 7 on the chromatograms. It had the R_f values shown in Table 2. It gives an intense green colour with ferric salts and a heavy reddish brown precipitate with boiling HCl. All the above properties show the substance to be (–)-epicatechin. This has previously been isolated from cacao by ether extraction (Freudenberg *et al.* 1932). A preparation isolated in the conventional manner showed identical properties with the above.

The mother liquors from the crystallization of (–)-epicatechin were combined. A large excess of (–)-epicatechin was still present. The catechins could be purified and partly separated by simply adding the aqueous solution to a column of wet cellulose pulp (12.5×3.7 cm.) and eluting with water. After 30 ml. of blank eluate had passed through 34 ml. was collected containing only coloured impurities followed by 120 ml. of a mixture of catechins and then 130 ml. containing only (–)-epicatechin. The catechin fractions were exhaustively extracted with ether and the ether evaporated. (–)-Epicatechin (67 mg.) and 72 mg. of the mixture were obtained. The mixture gave on crystallization from water mainly short prisms with a few long needles. Paper chromatography suggested that (–)-epicatechin and the other catechin were present in the mixture in the ratio 4/1 as judged from the intensity of the spots. Thus the maximum possible yield of the minor catechin would be 14.5 mg. Isolation in the presence of excess (–)-epicatechin was not successful. Incubation of the material with a dried preparation of mycelium of *Aspergillus niger* for 12 days failed to liberate any gallic acid and the chromatogram was unchanged. The same preparation readily split an authentic sample of (–)-epigallocatechin gallate (Bradfield *et al.* 1947). The substance is not, therefore, a catechin gallate ester. It was also stable to mild acid hydrolysis. From R_f values (Bradfield & Bate-Smith, 1950; Wender & Gage, 1949) the substance is provisionally identified as catechin itself. The rotation is not known.

Cut C. Paper chromatography showed that cut *C* consisted almost entirely of (–)-epicatechin. On crystallization from water 234 mg. of (–)-epicatechin were obtained. The mother liquor on concentration showed traces of another catechin which gave all the catechin reactions and was not a gallate ester. It could not be separated by paper chromatography from an authentic sample of (±)-gallocatechin. The substance was obviously present in too small quantity for isolation to be possible.

Cut D. From cut *D* 578 mg. of a light-brown powder was collected. On refluxing with 20% (w/v) HCl, or better ethanolic HCl, cyanidin is produced, confirmed by paper chromatography in butanol-2*N*-HCl and by colour tests.

The powder was very soluble in water. On paper chromatography of the solution, it was seen to be a mixture of (–)-epicatechin, gallocatechin, and spot no. 6 of Table 1. The powder was dissolved in 5 ml. methanol and precipitated with 45 ml. ether. The ether-soluble fraction was evaporated to give 145 mg. of a colourless solid. This material did not give cyanidin on treatment with HCl. Paper chromatography showed (–)-epicatechin, gallocatechin, and a third spot giving all the catechin reactions to be present. The mixture has not been separated. The chromatogram was unaffected by tannase. The unknown catechin could not be separated from (–)-epigallocatechin on chromatography. The three catechins were judged to be present in the proportion 3:1:3 from the intensity of the spots.

The ether-insoluble fraction was a light-brown amorphous powder (400 mg.) which could not be obtained crystalline and which darkened on heating, but did not melt below 300° . This material gave cyanidin on treatment with strong acid, but no sugar could be detected on milder hydrolysis. It did not give an immediate spot with ammoniacal AgNO_3 but only on standing.

Cut E. Cut *E* was treated as was cut *D*, 65 mg. were ether-soluble. The ether-insoluble material was the leucocyanidin as before (100 mg.). The ether-soluble material was a mixture of epigallocatechin and spot no. 4 of Table 1.

Cut F. Cut *F* consisted mainly of spot no. 4 in Table 1. It gave the usual catechin colour reactions and was unaffected by tannase. No crystalline products could be obtained.

Cut G. Cut *G* contained very little material which had the properties of spot no. 2 in Table 1. It gave the catechin colour reactions, but was unaffected by tannase.

DISCUSSION

The fresh cacao bean is seen to contain a very complicated mixture of polyphenolic substances. At least eleven different compounds can be distinguished by chromatography. The similarity between widely different varieties is striking.

The two main anthocyanins have been shown to contain respectively, glucose and arabinose, and glucose, we would therefore expect them to be cyanidin-3-arabinoglucoside and cyanidin-3-glucoside, i.e. chrysanthemin. However, the second anthocyanin is certainly not chrysanthemin (solubility of picrate), and an examination of colour reactions would suggest that the sugars are linked to a different hydroxyl (Robertson & Robinson, 1929).

To settle this point it would be necessary to isolate the pigments in large yield and compare them with synthetic preparations. Although up to 50% of the naturally occurring cyanidin glycosides may be of the 'pentose' glycoside (including methyl pentose) form (Robinson & Robinson, 1931, 1932) it is seldom known which pentose is present, and it is usually assumed that the pentose is rhamnose. An exception is sambucyanin (Nolan & Casey, 1931), which contains an aldopentose and glucose. Arabinose does not appear to have been identified previously in a cyanidin glycoside, although

Table 3. Comparison of cacao, and known cyanidin anthocyanins

Anthocyanin	Sugars	R_F Butanol- acetic acid	Colour at pH 8	Sugar linkage
Mekocyanin	Gentiobiose	0.29	Brown-red	3
Cacao anthocyanin II	Glucose	0.33	Blue-violet	?
Chrysanthemin	Glucose	0.33	Brown-red	3
Mildly hydrolysed anthocyanin I	Arabinose-glucose	0.35	Blue-violet	?
Antirrhinin	Rhamnose-glucose	0.37	Brown-cherry	3
Cacao anthocyanin I	Arabinose-glucose	0.41	Blue-violet	?
Cyanidin	—	0.69 (in butanol/ 2N-HCl)	Blue-violet	—

vicianose is a common constituent of other types of glycosides. The available information is summarized in Table 3.

At least two compounds of the leucoanthocyanin class are present, both based on cyanidin. One would appear to be a leucocyanidin and the other (no. 1) a leucocyanidin glycoside. The second is probably a mixture of different glycosides based on both arabinose and glucose. Attempts are being made to isolate the leucocyanidin in large yield for structural investigation.

At least six of the polyphenols have the properties of catechins of which the only one present in quantity (at least 40% of the total ethyl acetate-soluble 'tannins') is the previously isolated (—)-epicatechin. No evidence of gallates was encountered. A comparison of the catechins isolated from cacao and tea (Bradfield *et al.* 1947; Bradfield & Penney, 1948) has led to the provisional identification of catechin, galocatechin, and epigallocatechin, by chromatography. The other two catechins are unidentified.

SUMMARY

1. The polyphenolic components in extracts of fresh cacao beans have been examined by paper strip chromatography.

2. At least eleven polyphenols are present and have been separated on columns of cellulose pulp.

3. In Forastero (purple) beans there are two main anthocyanins with also a trace of a third pigment, with the properties of a cyanidin diglycoside. The two main pigments are a cyanidin monoglycoside and a cyanidin pentose glycoside, the sugar radicals being respectively glucose and glucose-arabinose.

4. A sugar-free leucocyanidin and a mixture of leucocyanidin glycosides are also present.

5. There are at least six catechin-like substances of which epicatechin is the main component. From chromatographic comparison, catechin, epigallocatechin and galocatechin, would appear to be present.

6. The chemical similarity between different species and varieties of beans used in chocolate manufacture is striking.

I am indebted to Dr A. E. Bradfield for supplying the samples of galocatechin, epigallocatechin, and epigallocatechin gallate which were used in the chromatographic work, to Dr A. C. Thaysen, Director, for his interest in this work, and to the Colonial Products Research Council for permission to publish.

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Cacao Polyphenolic Substances

2. CHANGES DURING FERMENTATION

BY W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Fresh cacao beans are prepared for marketing by heaping in boxes so that their covering of mucilaginous pulp can be broken down through the action of yeasts and acetic acid bacteria (Knapp, 1937). After this fermentation the beans are sun-dried. During both processes various changes take place in the cotyledons of the beans, which result in a product suitable for the manufacture of chocolate. The most striking chemical changes taking place during processing of fresh cacao beans are the alterations in the polyphenolic substances of the beans, the simpler polyphenols yielding insoluble complexes (Hallas, 1939, 1949).

From analogy with tea fermentation, it has generally been assumed (Knapp, 1937) that the initial change in the cacao bean fermentation is one of oxidation by atmospheric oxygen activated by a polyphenol oxidase system. Oxidases are certainly present in abundance (Brill, 1915; Ciferri, 1931).

In previous work in this series (Forsyth, 1949, 1952) it has been shown that the polyphenols extracted from fresh Forastero cacao with dilute acid consist mainly of catechin and cyanidin compounds. Both anthocyanins and leucoanthocyanins are present. Of the catechins, epicatechin is present in by far the greater proportion.

By quantitative paper chromatography of extracts of beans removed from the fermentation heap throughout the process, the destruction and conversion of the main polyphenols has now been followed. The changes during artificial treatments of the beans have also been investigated.

peeled and the cotyledons (about 20 g.) immediately extracted with 0.3N-HCl as described in Part 1 (Forsyth, 1952).

Chromatographic estimation of polyphenols. The extract (0.2 ml.) was streaked across the starting line (16 cm.) of a Whatman no. 1 paper sheet 20 cm. wide. Two lanes at the

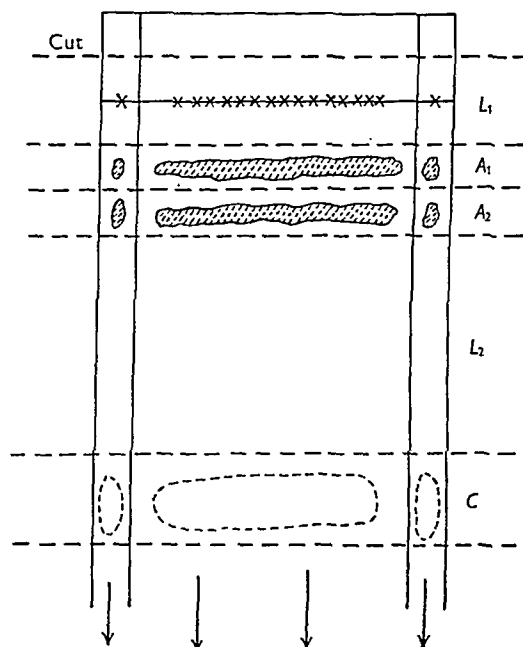


Fig. 1. Quantitative paper chromatography of 0.3N-HCl extract of fresh cacao beans. L_1 , complex leucocyanidins; L_2 , leucocyanidin; A_1 , cyanidin monoglucoside; A_2 , cyanidin arabinoglucoside; C , epicatechin.

METHODS

Commercially fermented beans. Twenty beans were taken at random from a fermentation 'sweat box'. This number is required to give a representative sample. The beans were

side were used to spot on marker solutions of fresh bean extract. The sheets were then chromatographed with amyl alcohol-acetic acid-water for 18 hr. (Forsyth, 1949, 1952) by descending chromatography. Before drying the paper the anthocyanin pigments were cut out and extracted to pre-

vent fading. The remainder of the paper was allowed to dry at room temperature. The marker strips were cut out and passed through vanillin-HCl to detect the epicatechin spot. The sheets were cut as shown in Fig. 1. In all, five components were estimated which make up the bulk of the soluble polyphenols of Forastero cacao beans.

The leucocyanidins (L_1 , L_2). These compounds were not detected on the paper, but were known to be present in the selected sections of the chromatogram (Forsyth, 1952), along with traces of the other catechins. They were estimated by refluxing the paper sections for 15 min. with 10 ml. *n*-butanol and 2 ml. 10*N*-HCl which converts them to cyanidin. The cyanidin solution was purified and determined colorimetrically as described by Hallas (1949). Blank paper sections gave no coloration with this treatment.

The anthocyanins (A_1 , A_2). These pigments were extracted from the paper with 10 ml. methanolic HCl (0.3*N*) and estimated colorimetrically (Hallas, 1949).

(-)-*Epicatechin (C)*. The catechin was detected with vanillin-HCl and estimated by extraction with 10 ml. 1% (v/v) H_2SO_4 and titration with 0.01 *N*- $KMnO_4$.

As controls, fresh beans were used, extracted and chromatographed simultaneously and in an identical manner. All results are expressed as a percentage of each component as present in the fresh bean. An HCl (0.3*N*) extract is stable for at least 24 hr. since, although the enzymes do not appear to be destroyed by the acid, they do not act at this low pH.

Laboratory treatment of beans for the study of oxidase activity

Grinding in air. When fresh, peeled beans are ground in a mortar and exposed to the air a rapid browning and complete disappearance of all the soluble polyphenols present takes place within 1 hr. After only 15 min. grinding over 80% of each component has been destroyed. Such a treatment is not suitable for enzyme studies. It was found, however, that similar extensive changes take place when beans are disintegrated in water, if sufficient aeration is permitted.

Grinding in aqueous solutions. Twenty fresh peeled beans were disintegrated in a Waring Blendor for 1 min. with 100 ml. water, left for 4 min., reblended for 0.5 min., left for 4.5 min., reblended for 0.5 min., left for 4.5 min., and then the acidity of the suspension made 0.3*N* with conc. HCl and blended for a further minute. The final acid suspension was filtered and the polyphenols remaining determined in this filtrate by the previously described method. In this way, the beans were exposed for 15 min. to high aeration and disintegration. Boiled beans treated similarly showed no change. The pH during the blending could be made any desired value, and enzymic poisons could be introduced, to study their effect on the oxidase system. To study the effect of temperature the beans could also be blended in hot water, maintained at 50° by immersing the blender in a water bath between the short blends.

Determination of oxidase activity in whole beans. Although cacao beans contain a particularly powerful oxidase system the results from a commercial fermentation did not suggest that this system is acting in the cotyledons. This could only be due to the absence of an adequate supply of oxygen gaining access to the cotyledons during commercial fermentation. To test this, fresh pulp-free beans were fermented in anaerobic jars packed with cellulose pulp containing buffer pH 5.5 for 5 days at 48°, conditions similar to

those in a sweat box but under completely sterile conditions, and in a hydrogen atmosphere.

The beans were extracted with acid in the normal manner, but the polyphenols in the extract from both peeled and unpeeled beans were estimated to determine the losses due to exudation of polyphenols, from the cotyledons to the tests.

Laboratory treatment of beans for the study of anaerobic enzyme activity

The above methods are not very suitable for a more intensive study of the enzymes since it would be difficult to obtain a disintegrated suspension of the beans without oxidation taking place. It was therefore decided to investigate whether a dry powder could be obtained containing the enzymes and substrate intact. This is feasible, since in fresh beans all the polyphenolic substances are located in the vacuoles of special isolated cells, and if the beans are washed free of pulp and dried in the sun, or dehydrated at low temperatures in a well ventilated oven, the cells are dehydrated *in situ*, and no change in the solubility of the components can be detected. The dry beans can then be ground to a fine powder without enzyme action taking place owing to their low moisture content. It was found that such a powder serves as a reasonably stable source of enzyme and substrate.

To investigate the anaerobic changes dry powder (20 g.) was submerged in water (100 ml.) sealed with liquid paraffin, for various times. The powder wetted easily and only a preliminary shake to mix was given. The suspension was then acidified to 0.3*N* with HCl as before, blended for 3 min., and filtered. The polyphenols were estimated in the usual manner. The pH and temperature could be varied and enzyme poisons introduced. In no case was the catechin affected under anaerobic conditions, except where H_2O_2 was added, and the constancy of the catechin value was used as a check on the anaerobiosis. Any extract in which the catechin deviated from the control by more than 5% was discarded. Boiled beans were unaffected by this treatment.

RESULTS

In normal Trinidad practice the fresh beans are allowed to ferment in sweat boxes for from 6 to 8 days and are then removed to the drying platform for sun-drying. A typical analysis of such beans is shown in Fig. 2.

The effects of pH and of inhibitors on the changes activated by the oxidases, during blending, are shown in Table 1. When the beans were ground at 50° at pH 5.0 the oxidase system remained active, i.e. the oxidases were not to any great extent inhibited by the highest temperature and acidity reached in commercially fermented beans in the sweat box. However, when the residual epicatechin was determined in both the cotyledons and testa of whole unpeeled beans, there was no significant difference in amount between the catechin remaining in the cotyledons of beans, fermented for 5 days in the complete absence of external oxygen, and those commercially fermented. The loss of catechin (20–30%) must be due in both cases to exudation

from the cotyledons to the testa. This exuded catechin becomes partly oxidized in the testa of commercial beans but not, of course, in beans fermented under completely anaerobic conditions.

Anaerobic enzyme activity

That the dry powder used in the following experiments can serve as a source of enzymes and substrate comparable with the fresh beans is shown in Table 2. In this table is also included a test for peroxidase which is shown to be present but which does not act under anaerobic conditions unless hydrogen peroxide is added.

It will be seen in Table 2 that some changes take place in the cyanidin compounds under anaerobic conditions even at room temperature. These changes are much more marked at 48°. This temperature was chosen as being within the range of temperature prevailing in the sweat box at the time of killing of the bean. The result of incubating the powdered beans anaerobically at 48° at various pH values are shown in Figs. 3 and 4.

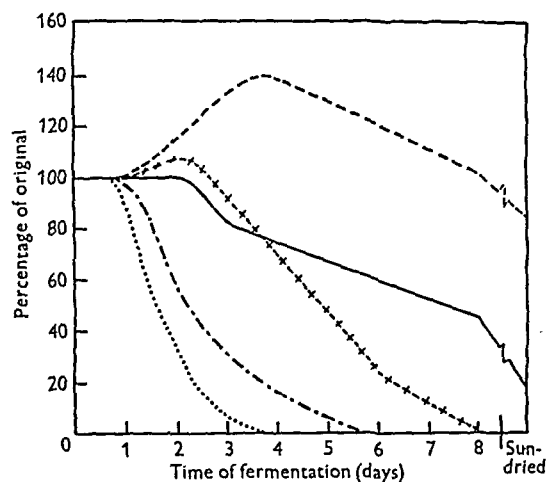


Fig. 2. Changes in soluble polyphenols during a commercial fermentation. Symbols as in Fig. 1;, A_1 ; ----, A_2 ; —, C ; ----, L_1 ; -+ -+, L_2 . Sun-dried refers to beans which, after undergoing the commercial fermentation, were exposed to the sun till dry, i.e. the commercial product as shipped.

Table 1. *Oxidase activity of fresh bean homogenates in water, various buffers (MacIlvaine's) and in the presence of inhibitors*

(For conditions of blending and method of estimation see text. The inhibitor was added before disintegration of the beans. Results as percentage of original amount of each component remaining after 15 min.)

Solution	pH	Cyanidin monoglucoside	Cyanidin arabinoglucoside	Complex leuco-cyanidins	Leuco-cyanidin	Epicatechin
Water	—	<5	<5	17	<5	13
	5.5	<5	13	7	<5	18
MacIlvaine's buffer	5.0	10	8	12	<5	21
	4.5	12	10	16	10	35
	4.0	55	54	58	72	80
10 ⁻² M-KCN	5.5	52	50	80	60	71
10 ⁻² M-KCN	5.5	103	107	100	94	101
10 ⁻¹ M-Ascorbic acid	5.5	72	67	101	74	102

Table 2. *Comparison between fresh beans, sun-dried beans, and sun-dried powdered beans*

(For methods of estimation see text. Results as percentage of each component in original fresh beans.)

Test for	Preliminary treatment	Cyanidin monoglucoside	Cyanidin arabinoglucoside	Complex leuco-cyanidin	Leuco-cyanidin	Epicatechin
Substrate	Sun-dried beans	91	94	110	97	94
	Sun-dried powdered beans	108	110	92	105	101
Oxidase activity	Fresh beans, blended water for 15 min. at 25°	<5	<5	15	<5	11
	Dry powdered beans blended as with fresh	<5	<5	32	<5	21
Anaerobic enzymic activity	Fresh beans killed by freezing and incubated in water anaerobically at 25° for 18 hr.	55	78	107	97	99
	Dry powdered beans treated as with frozen beans	45	87	115	87	102
Peroxidase	Dry powdered beans incubated anaerobically as above in 0.1M-H ₂ O ₂	33	69	59	42	58

Whole beans killed by preliminary freezing showed the same changes when incubated anaerobically as do powdered beans. The pH of 6.2 was chosen as being about the pH prevailing at the time of the death of the bean and pH 5.5 as covering the latter part of the fermentation.

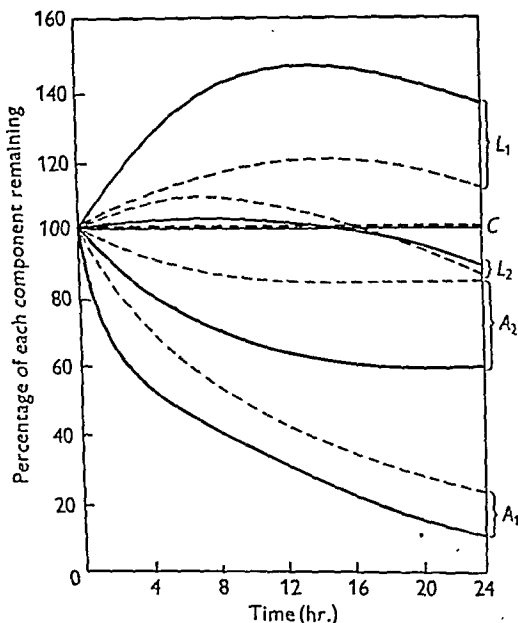


Fig. 3. Conversion of polyphenols in sun-dried powder at 48° under anaerobic conditions. Symbols as in Fig. 1; —, pH 6.2; ---, pH 5.5. 20 g. powder suspended in 100 ml. MacIlvaine's buffer under a paraffin seal for various times and the changes estimated as described in the text.

The effect of inhibitors on the anaerobic reactions is shown in Table 3. Sodium fluoride and copper sulphate appear to inhibit the formation of the complex leucocyanidins (L_1) while not preventing the apparent conversion of the anthocyanins to leucocyanidin (L_2).

In no case was cyanidin itself found on any of the chromatograms indicating that a simple hydrolytic mechanism was unlikely.

Table 3. *Effect of inhibitors on the anaerobic enzymic activity of sun-dried powdered beans*

(Powder (20 g.) was suspended in MacIlvaine's buffer pH 6.0 (100 ml.), sealed with liquid paraffin and maintained at 48° for 18 hr. then estimated as described in text.)

	Cyanidin mono-glucoside	Cyanidin arabinoglucoside	Complex leuco-cyanidins	Leuco-cyanidin
Boiled solution	92	95	100	98
Buffer	20	70	150	104
NaF ($10^{-2}M$)	15	64	72	130
CuSO ₄ ($10^{-2}M$)	33	62	58	132
KCN ($10^{-2}M$)	30	67	115	96

DISCUSSION

It has been generally assumed that the main change in the cacao polyphenols during fermentation is one of oxidation by a polyphenol oxidase system requiring external oxygen.

The beans certainly contain a particularly powerful polyphenol oxidase system capable of removing over 80 % of the total polyphenols in 15 min. when fresh beans are disintegrated and strongly aerated in buffer solutions, at the temperature and acidity prevailing in the sweat box. Peroxidase is also present. The oxidase has a high resistance to potassium cyanide in common with tea polyphenol oxidases (Lamb & Sreerangachar, 1940). However, during the commercial fermentation of the whole cacao beans no such rapid removal of the poly-

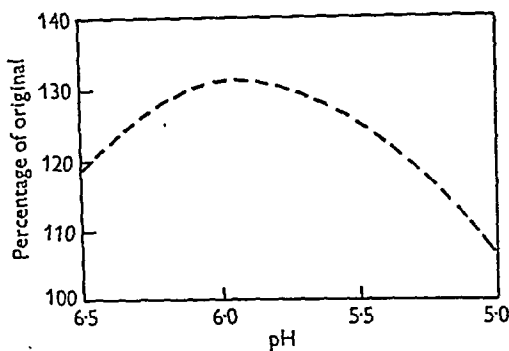


Fig. 4. The percentage of the complex leucocyanidin fraction (L_1) remaining after incubation of sun-dried powder at various pH values in MacIlvaine's buffers anaerobically for 5 hr. at 48°. (Amounts used as in Fig. 3.)

phenols takes place. Instead the simple cyanidin compounds are destroyed and more complex leucocyanidins are formed. The catechin is slowly removed from the cotyledons. When powdered beans are fermented under anaerobic conditions similar changes take place in the cyanidin compounds, but the catechin is completely unaffected. By fermenting whole beans under anaerobic conditions, it can be shown that the loss of catechin in commercial fermentation is due entirely to its exudation from the cotyledons to the testa.

The lack of activity of the oxidase system in the cotyledons during a commercial fermentation can only be due to a restriction in the supply of available oxygen. Such lack of oxygen is not surprising. In fermenting beans the microflora of the pulp will most certainly consume most of the oxygen introduced into the heap by ventilation. This is supported by the observation that fermenting pulp decolorizes methylene blue. Further, even in pulp-free aseptically fermented beans, the cotyledons resist oxidation. This is due to the changes which take place in the beans about the second day, when they are killed by the temperature and acidity. The free space between the cotyledons and the testa then becomes completely filled with a continuous layer of purplish juice. Paper chromatography shows that this juice contains all the soluble components of the cotyledons and that it is especially rich in epicatechin. In a commercial fermentation this juice is partly absorbed by the testa and some is even lost in the pulp and sweatings. The exudate and the testa themselves undergo browning during fermentation, but even in fully fermented beans the juice still contains considerable catechin and acts as a further barrier of oxidizable substances and prevents access of air to the cotyledons. Oxidation in the cotyledons (i.e. the part used in chocolate manufacture) only takes place during the drying period.

The significant change during fermentation would thus appear to be the conversion of the cyanidin compounds into more complex products by an enzyme system acting independently of external oxygen. This conversion has an optimum pH of about 6.0, the mean pH of the fermenting

bean on the first day after the death of the bean. When the reaction is carried out in the presence of enzyme poisons the conversion of pigments to leuco compounds and the subsequent condensation of the latter can be differentiated.

It is tempting to suggest that this conversion of cyanidin compounds may be of greater importance than the previously postulated 'oxidation of tannins' in determining the flavour and aroma of the final product.

SUMMARY

1. The changes in the polyphenolic constituents of cacao cotyledons during commercial fermentation have been estimated by quantitative paper chromatography.

2. The main change is the conversion of the simple cyanidin compounds to more complex leuco-cyanidins.

3. Although oxidases are present they do not act in the cotyledons during fermentation due to the anaerobic conditions prevailing.

4. The catechin is partly lost by exudation and is then to some extent oxidized under the more aerobic conditions prevailing in the testa. Oxidation in the cotyledons, however, only takes place during the drying period.

5. It is possible to obtain conversion of the cyanidin compounds with sun-dried, unfermented, powdered beans, in buffers under anaerobic conditions. The reaction appears to consist of several stages.

The author is indebted to Dr A. C. Thaysen, Director, for his interest in this work, and to the Colonial Products Research Council for permission to publish.

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Distribution of Copper and Zinc in Mammalian Eyes. Occurrence of Metals in Melanin Fractions from Eye Tissues

By J. M. BOWNESS, R. A. MORTON, M. H. SHAKIR AND A. L. STUBBS
Biochemistry Department, The University of Liverpool

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Several methods have been used in the investigation of trace elements in eye tissues. Burdon-Cooper (1928) and Burdon-Cooper & Lewis (1929) give the results of emission spectrographic analysis of the mineral constituents of normal and cataractous human-eye lenses. The technique failed to demonstrate the presence of zinc, iron and manganese, which Tauber & Krause (1943) were later able to detect (and estimate) by other methods. The latter authors pointed out that the persistent spectral lines of the three elements could easily be obscured by lines due to elements such as calcium and phosphorus, which are present in the ash as major constituents.

The first stage in the present investigation was the application of modern methods of emission spectrum analysis to the problem of detecting and determining trace metals in eye tissues (A.L.S. and M.H.S., see Shakir, 1948). So far as detection was concerned, excellent results were obtained, but the range covered by zinc, copper and manganese contents of various eye tissues proved to be too narrow for emission spectra to give quantitative information of sufficient accuracy. Accordingly, a good deal of exploratory work was carried out using organic reagents forming metallic complexes which could be estimated by spectrophotometric methods. Selected procedures have now been improved and the results revised and extended (J.M.B.).

In the course of the work based on emission spectroscopy, semi-quantitative estimations of trace elements were made on dialysed and undialysed tissues from fish eyes (Shakir, 1948). The presence of comparatively large quantities of dialysable inorganic material results in a cloud of vaporized salts around the electrodes, and this has a marked effect on the recorded intensities of the spectral lines shown by the 'trace' elements. This effect introduces uncertainty into the validity of comparing the results with dialysed and undialysed preparations. A fivefold difference in trace metal concentration is certainly demonstrable, but it is difficult to be sure about small differences. The emission spectrum technique is thus not sufficiently sensitive for further study of the rather small differences recorded by Tauber & Krause (1943) in respect of the concentrations of zinc, copper and

manganese in cattle-eye tissues. In spite of such limitations, it seemed clear that tissues from fish eyes contained non-dialysable zinc and copper in excess of the retained amounts of other trace metals. The preliminary work with dithizone zinc and copper complexes indicated that it might be possible to establish significant differences between the various eye tissues in mammals. Repetition and extension of the work shows this to be the case.

Leiner & Leiner (1944) had shown for zinc in fish eyes that such differences occur. Although the absolute concentrations found in each tissue varied greatly from species to species, a list showing the different types of tissues in order of decreasing zinc content was practically the same for all the fishes studied.

The results of Tauber & Krause (1943) on cattle eyes do not show a similar distribution of zinc, but the analytical method described in their paper seems inadequate. Their determinations of copper concentrations in eye tissues appear to be the only ones recorded in the literature.

It was therefore decided that the whole problem of zinc and copper in eye tissues deserved further study. The first part of this paper embodies the experimental details and results of determinations of zinc and copper concentrations in all the eye tissues of several mammals. The results of these determinations show that the iris and choroid tissues contain the highest concentrations of zinc and copper. It therefore became necessary to investigate the more precise location of these metals in irises and choroids. The second part of the paper deals with the experimental methods and results of a fractionation of irises, and a study of a pigment fraction obtained from irises and choroids.

1. ZINC AND COPPER CONCENTRATIONS IN THE VARIOUS EYE TISSUES

EXPERIMENTAL

Materials

Cattle and sheep eyes were obtained from the abattoir and dissected within 12 hr. of the death of the animals.

Whale eyes were obtained from sperm whales caught in the Antarctic region, and had been stored (whaling ship

'*Balaena*') in tins at a temperature below 0° for several months before dissection.

Rabbit eyes from animals of Dutch, cross-bred, Himalayan and albino varieties, were obtained through the courtesy of Dr A. U. Smith, from the National Institute for Medical Research, Mill Hill, London, N.W. 7, and were dissected 2-3 days after the death of the animals.

Analytical methods

General. Concentrations of Zn and Cu were determined mainly on a dry weight basis, for the tissues can be lightly washed, to remove adhering fluids and particles, with very little alterations in solid content, though the natural wet weight cannot then be accurately determined. Approximate wet weights of sheep and cattle tissues were obtained by drying the tissues, after washing, with ashless filter paper. A comparison of wet and dry weight concentrations of the trace metals then enables the effect of the loss of water from liquid tissues, such as the vitreous humour, to be gauged.

Preparation of tissues for analysis. Dissection was carried out with stainless steel instruments. An incision in the sclera was made with a scalpel and the front portion of the eye parted from the back by cutting round the outer margin of the iris. The tissues of the two portions were then separated and the two parts of the sclera combined, after removing the adhering muscle and fatty tissue. Each individual tissue was then washed with twice-distilled water. All manipulations after washing were carried out with glass rods.

The tissues were dried to constant weight in an oven maintained at 110° .

Tissues were normally incinerated in translucent silica crucibles at $450-550^{\circ}$ in an electric muffle furnace with a silica lining. If the last trace of carbon residue was difficult to burn away it was found that addition of a few drops of twice-distilled water to the cooled ash permitted easy oxidation of the carbon on reheating. Lens tissue must be very slowly heated to 450° , otherwise the contents of the crucible froth over.

To each crucible containing ash, $0.1\text{ N-H}_2\text{SO}_4$ (10 ml.) was added and the contents evaporated to dryness on a steam bath. More $0.1\text{ N-H}_2\text{SO}_4$ (5 ml.) was then added and the crucible heated on the steam bath for a further 10-15 min. The contents were then washed into the beaker used to support the crucible in the extraction procedure, and the solution was made up to a standard volume ready for the estimation of Cu.

Analytical method. Both Cu and Zn were estimated colorimetrically by means of diphenylthiocarbazone (dithizone) solution in CCl_4 . From an acid solution only the Cu complex is extracted; if this is first removed, Zn can then be estimated by buffering to pH 4.75, and again extracting with dithizone. Extinction coefficients of the CCl_4 solutions were measured by means of a Beckman photoelectric spectrophotometer.

The method given by Sandell (1944) was used for Cu, and a modification of the method of Vallee & Gibson (1948) for Zn.

The absorption curve of dithizone in CCl_4 solution (Fig. 1) has a minimum about $510\text{ m}\mu$, and at this wavelength the curve for copper dithizonate (Fig. 2) is near its maximum. Thus, extraction with a known excess of dithizone, and measurements of the extinction coefficient at $510\text{ m}\mu$, will

indicate the amount of Cu present. This method is not practicable for Zn because a large excess of dithizone is needed for the Zn to be extracted quantitatively. The extinction coefficient of pure zinc dithizonate at $620\text{ m}\mu$ is very low indeed (Fig. 3) and thus, in a solution containing both dithizone and zinc dithizonate, measurement of the E value at $620\text{ m}\mu$ gives a measure of the excess dithizone present. Zinc dithizonate has a maximum at $535\text{ m}\mu$ (Fig. 3) and thus, if the extinction at this wavelength is measured, and the contribution made by excess dithizone to the total absorption at $535\text{ m}\mu$ is calculated and subtracted, the Zn can be estimated. (The relative E values for pure dithizone at 620 and $535\text{ m}\mu$ are known (Fig. 1), so that measurement at $620\text{ m}\mu$ allows the E value at $535\text{ m}\mu$ to be computed).

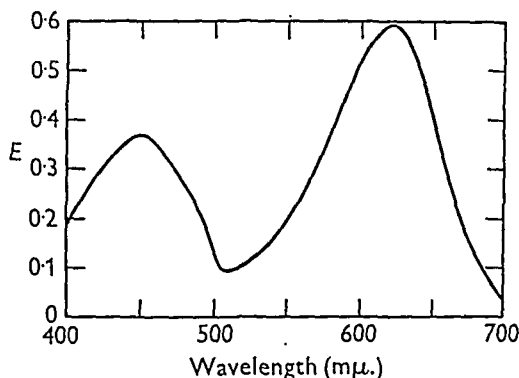


Fig. 1. Absorption spectrum of diphenylthiocarbazone in CCl_4 . $E_1^{1\%}$ at $\lambda_{620\text{ m}\mu} = 540$.

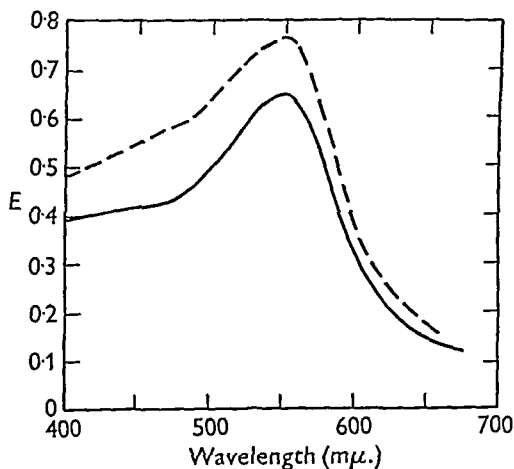


Fig. 2. Absorption spectra of copper dithizonate solutions in CCl_4 . —, ash from eye tissues dissolved in $0.1\text{ N-H}_2\text{SO}_4$. Excess of this solution extracted with dithizone in CCl_4 . ---, excess CuSO_4 in $0.1\text{ N-H}_2\text{SO}_4$ extracted with dithizone in CCl_4 (about $0.1\text{ }\mu\text{g. Cu/ml.}$).

Reagents and apparatus. Twice-distilled water (the final distillation being from, and into, Pyrex glass vessels) was used to make up all solutions, and for the final washing of the vessels. All glass vessels used were of Pyrex and, after washing, were dried in an oven at 110° and stored away from dust.

All reagents used were of standard A.R. grade except diphenylthiocarbazone (British Drug Houses Ltd.) which bore no special label:

(a) $0.1\text{N-H}_2\text{SO}_4$: 2.7 ml. A.R. H_2SO_4 made up to 1 l.

(b) Sodium thiosulphate solution: 25 g. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in 100 ml. water.

(c) Buffer (pH 4.75): 2N-acetic acid (500 ml.) and 2N-sodium acetate (500 ml.) were mixed together. Even when reagents of A.R. quality were used, this solution gave a substantial 'blank' reading in the Zn estimation. This was eliminated by shaking 1 l. of the buffer solution, for 5–10 min. each time, with successive portions of 0.01% dithizone solution, and discarding the lower (CCl_4) layers until the colour of the dithizone remained unchanged.

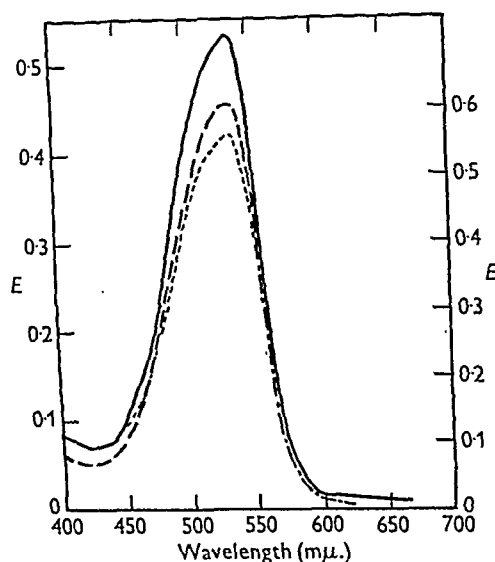


Fig. 3. Absorption spectra of zinc dithizonate solutions in CCl_4 . —, absorption spectrum of zinc dithizonate in CCl_4 (about $0.35\text{ }\mu\text{g. Zn/ml.}$) — — —, ash solution from choroid (pH 4.75) extracted with dithizone in CCl_4 . Absorption curve determined and corrected for excess dithizone (peak at $620\text{ m}\mu$.) - - - -, ash from iris treated similarly. The curve for pure zinc dithizonate corresponds with the ordinates on the left, the other curves with those on the right.

(d) Copper solutions: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1964 g.) was dissolved in $0.1\text{N-H}_2\text{SO}_4$ (1 l.). From this a solution containing $1\text{ }\mu\text{g. Cu/ml.}$ was prepared by dilution with $0.1\text{N-H}_2\text{SO}_4$.

(e) Zinc solutions: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2198 g.) was dissolved in $0.1\text{N-H}_2\text{SO}_4$ (1 l.). From this a solution containing $1\text{ }\mu\text{g. Zn/ml.}$ of $0.1\text{N-H}_2\text{SO}_4$ was prepared by dilution.

(f) Dithizone solutions (0.01 and 0.003% w/v) were made up in A.R. CCl_4 and filtered. These solutions deteriorate slowly as a result of oxidation. Fresh solutions were made up every 3 months and stored in the dark at 0° .

Calibration. (i) *Copper*: solutions containing $0\text{--}10\text{ }\mu\text{g.}$ of metal in 10 ml. $0.1\text{N-H}_2\text{SO}_4$ were extracted with 0.003% dithizone solution (6 ml.) in separating funnels. The lower layer was run off into a 10 ml. standard flask and the remaining drops washed through with CCl_4 . Readings of the extinction at a wavelength of $510\text{ m}\mu$ were made on the spectrophotometer. A straight-line graph was produced by

plotting the extinction against amount of Cu to be extracted. A similar graph was produced for a $0\text{--}25\text{ }\mu\text{g.}$ range by using proportionately greater quantities of reagents and final dilution of the CCl_4 extract.

(ii) *Zinc*: solutions containing $0\text{--}10\text{ }\mu\text{g.}$ of metal in 10 ml. $0.1\text{N-H}_2\text{SO}_4$ were placed in separating funnels and 5 ml. buffer (pH 4.75) and 1 ml. sodium thiosulphate solution were added. Each mixture was then shaken with successive 1 ml. portions of 0.01% dithizone solutions until the lower layer (CCl_4) remained green after shaking for 2 min. Each portion was run off into a 20 ml. standard flask and the last drops were washed through with CCl_4 . The solution was made up to volume and readings of extinctions at 535 and $620\text{ m}\mu$ were made.

A straight-line graph was produced by plotting $E_{535\text{ m}\mu}$ — $0.25\text{ }E_{620\text{ m}\mu}$ against the amount of Zn ($0\text{--}10\text{ }\mu\text{g.}$) to be extracted. A similar graph was obtained for the range $0\text{--}50\text{ }\mu\text{g. Zn}$, after diluting the final extract to 50 ml.

Blank estimations were carried out with each set of Zn and Cu estimations as a precaution against casual contamination in the apparatus and standard solutions; these estimations also served as a check on the deterioration of the 0.003% dithizone solution used to extract Cu. If the blank estimations were found to give a lower extinction than when the dithizone solution was fresh, the standard graph was recalibrated. The $E_{620\text{ m}\mu}/E_{535\text{ m}\mu}$ ratio of the 0.01% dithizone solution was checked weekly, and the current value was used in calculating the absorption at $535\text{ m}\mu$ due to dithizone. The average value for the ratio was 4.0 and the greatest drop recorded in 3 months was from 4.2 to 3.8.

Specificity of the extraction procedures. Sandell (1944) states that several other metals are partially extracted, if present in certain relative concentrations, by the procedures used here for Cu and Zn. To determine whether such metals are present in amounts sufficient to cause significant interference in the present work, absorption curves for extracts from typical ash preparations of eye tissues were obtained by the above procedures.

In the case of Cu, all the dithizone shaken up with a solution of Cu salt in $0.1\text{N-H}_2\text{SO}_4$ is converted to copper dithizonate merely by the presence of a sufficient excess of Cu in the aqueous phase. Thus Fig. 2 compares the absorption curve of dithizone in CCl_4 shaken up with large excesses of CuSO_4 solution, and a solution of mixed eye-tissue ash, respectively.

In the case of Zn, excess dithizone is always present in the extract. However, if it is assumed that the absorption of a zinc dithizonate solution at $620\text{ m}\mu$ is zero (which is very nearly the case), then, if Zn is the only metal extracted, the absorption at $620\text{ m}\mu$ is solely due to dithizone. From the absorption curve for pure dithizone the relations between the $E_{620\text{ m}\mu}$ value and the E values of a dithizone solution at other wavelengths is known, and thus the amount of light absorption at any wavelength due to dithizone in a mixed solution of zinc dithizonate and dithizone, can be found. By subtraction of the E values calculated to be due to dithizone, from the total E values at various wavelengths of such a mixture of dithizone and zinc dithizonate, the extinction values which should be due to zinc dithizonate, if the extraction procedure is specific, can be found. The absorption curve, plotted from E values obtained in this way for an extract of a typical eye-tissue ash, is given in Fig. 3. There is an almost exact correspondence between this calculated curve and a pure zinc dithizonate curve, obtained by decomposing the excess dithizone with Na_2S solution, in an

extract from a solution of ZnSO_4 under the standard conditions described above. Such a correspondence would hardly occur if another metal dithizonate were present, even though the original assumption, that the 620 $\text{m}\mu$. absorption was wholly due to dithizone, was unjustified.

The correspondence between the Cu curves demonstrates that here, also, the extraction procedure is specific for Cu.

The curves, both for Zn and Cu, agree with those given by Fischer & Weyl (1935) and Fischer (1937) for the pure metal dithizonates.

Estimation procedure. The solution of ash in 0.1 N- H_2SO_4 , obtained as described in the section on preparation of tissues for analysis, was made up to a standard volume with 0.1 N- H_2SO_4 (the exact volume depending on the weight and kind of tissue analysed) and a sample taken for Cu extraction. The sample was made up to approximately 10 ml. with 0.1 N- H_2SO_4 and shaken with 6 ml. of 0.003% dithizone solution in a separating funnel. If the CCl_4 layer was then red, a further 9 ml. of dithizone solution were added and the whole again shaken. The extract, either 6 or 15 ml. in volume, was then run off and made up to volume as described before. Comparison of the value of the extinction at 510 $\text{m}\mu$. with one of the standard graphs then indicated the amount of Cu present.

A smaller sample was taken for Zn estimation. This was first made up to approximately 10 ml. with 0.1 N- H_2SO_4 in a separating funnel and extracted with excess dithizone solution to remove Cu. Buffer (pH 4.75) and sodium thio-sulphate solutions were then added and Zn extracted as described in the section on calibration. The extract was washed through with CCl_4 and made up to a volume putting the extinction values within the effective range of the spectrophotometer. The amount of Zn present was obtained by referring to the standard graphs.

Accuracy of the method. The reproducibility of the results was tested by homogenizing a mixture of cattle irises, choroids and lenses in a Waring Blender. Portions of the homogenate were dried, reduced to ash and the Zn and Cu

estimated by the standard procedure given above. Dry weights of the homogenate portions were of the order of 0.5 g., which was a rough average figure for the dry weights of the whole tissues taken for analysis in the following work.

RESULTS

The results are summarized in Tables 1-4. In Table 1, samples 1-6, inclusive, were ashed in silica crucibles, and samples 7 and 8 in platinum crucibles. When tissues containing little organic matter, such as the aqueous and vitreous humours, were ashed,

Table 1. *Reproducibility of zinc and copper estimations on eight equal portions of a homogenate of cattle lenses, irises and choroids*

Sample	Zinc ($\mu\text{g./g.}$ dry material)	Copper ($\mu\text{g./g.}$ dry material)
1	168	87
2	161	90
3	159	85
4	160	81
5	154	86
6	148	80
7*	170	92
8*	160	85
Mean	160	86
Standard deviation	7.03	3.80

* Ashed in Pt crucibles; all others in silica crucibles.

the deviation from the mean for samples in silica crucibles was somewhat greater than that shown above, and the mean of the results for these samples was consistently less than that for samples ashed in platinum crucibles. This effect is presumably due to

Table 2. *Concentration of zinc in the eye tissues of some mammals*

(A dash indicates that no analysis was made. Numbers in brackets denote $\mu\text{g./g.}$ wet tissue.)

Tissue	Cattle ($\mu\text{g./g.}$ dry tissue)	Sheep ($\mu\text{g./g.}$ dry tissue)	Sperm whale ($\mu\text{g./g.}$ dry tissue)	Rabbit	
				Coloured ($\mu\text{g./g.}$ dry tissue)	Albino ($\mu\text{g./g.}$ dry tissue)
Iris plus ciliary body	246 (41.0)	436 (65.1)	99.5	127	54.4
Choroid plus pigment epithelium	139 (26.5)	277 (69.2)	37.2	466	86.2
Retina minus pigment epithelium	71.0 (7.2)	80.0 (7.3)	54.1	—	—
Lens	37.3 (15.0)	117 (47.2)	35.2	15.8	12.5
Aqueous humour	30.0 (0.29)	—	—	—	—
Vitreous humour	26.4 (0.35)	23.2 (0.29)	10.5	—	—
Sclera	14.6 (4.1)	56.0 (16.1)	0.33	—	—
Cornea	13.5 (2.3)	25.0 (3.6)	35.3	6.6	12.1
Optic nerve	6.8 (2.2)	—	—	—	—

Table 3. Concentrations of copper in the eye tissues of some mammals
(A dash indicates that no analysis was made. Numbers in brackets denote $\mu\text{g./g.}$ wet tissue.)

Tissue	Cattle ($\mu\text{g./g.}$ dry tissue)	Sheep ($\mu\text{g./g.}$ dry tissue)	Sperm whale ($\mu\text{g./g.}$ dry tissue)	Rabbit	
				Coloured ($\mu\text{g./g.}$ dry tissue)	Albino ($\mu\text{g./g.}$ dry tissue)
Iris plus ciliary body	27.5 (4.6)	50.1 (7.4)	5.9	11.6	14.7
Choroid plus pigment epithelium	9.8 (1.7)	13.5 (3.4)	2.2	16.8	21.0
Retina minus pigment epithelium	6.8 (0.67)	11.5 (1.3)	10.6	—	—
Lens	1.2 (0.46)	2.1 (0.8)	4.2	0.62	0.49
Aqueous humour	10.4 (0.10)	—	—	—	—
Vitreous humour	17.7 (0.24)	24.0 (0.3)	5.9	—	—
Sclera	4.8 (0.13)	5.1 (1.4)	0.074	—	—
Cornea	3.2 (0.57)	1.9 (0.26)	3.4	0.30	1.5
Optic nerve	5.6 (1.8)	7.9 (2.6)	—	—	—

the formation of stable silicates. (Only two platinum crucibles were available, so that silica crucibles were used for all analyses except where otherwise stated.)

Table 4. Zinc and copper concentrations in the eye tissues of three individual sheep

(Results are in $\mu\text{g./g.}$ dry tissue.)

Animal	Lens		Iris		Sclera	
	Cu	Zn	Cu	Zn	Cu	Zn
1	2.8	124	40	458	4.6	41
2	1.7	129	41	401	6.6	47
3	2.1	92	69	450	4.0	67

The results obtained for Cu in Table 1 are not indicative of the physiological Cu concentrations of the tissues used, for it has been observed that any aqueous solution placed in the Waring Blender accumulates Cu, presumably from the alloys of the stirring mechanism.

DISCUSSION

Table 2 shows that the zinc concentrations in the various types of tissue in mammalian eyes differ to a considerable extent. The differences are, in most cases, very much greater than could be accounted for by the experimental error (Table 1). The differences between the concentrations of copper in the various tissues, shown by the results of Table 3, are not so great, but in most cases are larger than the latitude, indicated in Table 1, which could arise from the experimental procedure. Table 4 shows that the differences in zinc and copper concentrations between the eyes of three individual sheep were

small compared with the differences between the various types of tissue of the same sheep. Variation between individuals of the same species was not studied further because the eyes were obtained from a large abattoir and it was not easy to ascertain the breed, or the history, of the animals.

That the differences between the various eye tissues go deeper than peculiarities either of individuals or species, is indicated by the fact that the tissues can be placed in an order (in respect of their zinc and copper concentrations) which is roughly the same in each of the species examined. This order is very similar to that obtained from the results of Leiner & Leiner (1944) on the zinc content of fish-eye tissues, though the absolute concentrations were often much higher than those in the mammalian species examined here.

The results given in Table 2 for zinc concentrations in cattle-eye tissues are very much higher than any of the values found by Tauber & Krause (1943) for bullocks. However, the specificity of their method of estimation is questionable. They extracted the dithizone complex from an aqueous solution, the pH of which was probably acid, and in any case was never rigorously controlled. The reaction of dithizone with zinc can be made specific only in a solution at about pH 4.75, and in the presence of sodium thiosulphate or some other suitable complex-forming reagent. In acid solution, without any added reagents, only copper is likely to be extracted. The zinc/copper ratio found by them is actually never much greater than 1, whereas the zinc content of most biological materials is several times as great as that of copper.

The distribution of zinc and copper between the various types of sheep-eye tissues coincides with that found by Shakir (1948), and the concentrations of zinc found in each case are very similar. The concentrations of copper shown in Table 3 tend to be somewhat lower than the results for corresponding tissues given by Tauber & Krause for cattle, and by Shakir for sheep; this difference may be due to individual variation of the animals and to variation in pasture food.

It is significant that, both in the present work on zinc and copper in mammalian eyes and in the work on zinc in fish eyes, the highest concentrations of the metals were found in the pigmented parts of the eye. It is relevant that the only species (of those examined here) in which the retina, or any other tissue, contains a higher concentration of either metal than the iris, or choroid plus pigment epithelium, is the whale, whose choroid is thick, spongy and low in pigment. In addition, the whale eyes had been preserved and, on dissection, it was found impossible to separate spots of adhering pigment epithelium from the almost liquid retina.

The results for coloured and albino rabbit eyes show without doubt that the high concentration of zinc in the iris and choroid tissues is dependent, at least partly, on the presence of melanin pigment. No large difference was observed between the copper concentrations in the two types of eye, but the total amount of copper estimated was so small in each case (0.8–2.0 $\mu\text{g.}$) that the error introduced by ashing makes the results probably not significant.

Flesch (1949) reported a connexion between the concentration of copper in rabbit hair, and pigmentation. He observed a small difference between the copper concentrations in white and black hair from the same rabbit, though white hair from one rabbit might contain more copper than the black hair from another. Fore (1950) found no difference in manganese contents of the two types of hair, but a big drop in ash weight in white hair. We have found a small lowering of zinc concentration in white hair, no difference in copper concentration, and again a big drop in ash weight. Thus, in the rabbit, it appears that though zinc and copper may be concerned in the pigment problem, some other mineral element or elements are present in enhanced quantity in pigmented hair.

2. FRACTIONATION OF PIGMENTED TISSUES

Flesch (1949) has shown that pigment separated from a mouse melanoma contains much more copper than the melanoma tissue itself. Leiner & Leiner (1944) showed that grey or black powders, separated by differential centrifugation from powdered dry fish eyes, contained rather more zinc

than colourless fractions, though a yellow fraction contained more still. They did not postulate a connexion between zinc concentration and pigmentation.

In view of these facts and the evidence recorded in the first part of this paper, it was decided to determine whether zinc and copper are concentrated in the pigment fraction of the iris and choroid tissues of mammals.

EXPERIMENTAL METHODS AND RESULTS

It was established by analysis that cattle irises and choroids from left and right eyes of the same animal differ in their zinc concentrations by less than 15% of the mean between the two, and in their copper concentrations by less than 20% of the mean.

A number of pairs of cattle eyes were obtained from the abattoir; one of each pair of irises dissected out was analysed whole, and the other of the pair fractionated to obtain pigmented material.

Fractionation

Whole irises (3–6) were digested with 5 ml. of an approximately 2% (w/v) trypsin suspension and about 25 ml. of twice-distilled water for 24–36 hr. at 37°. The melanin pigment can then almost all be washed away from the bulky residue of undigested fibrous, connective and muscular tissue, which is allowed to remain in the digestion flask.

Table 5. *Zinc and copper present in various fractions of cattle irises obtained by digestion with trypsin*

Fraction	Dry weight (g.)	Zinc ($\mu\text{g.}$)	Copper ($\mu\text{g.}$)
Exp. 1			
Undigested residue	0.0430	9.6	0.9
Combined washings	0.2679	24.6	8.0
Pigment fraction	0.1383	101.0	9.0
Total	0.4492	135.2	17.9
Trypsin blank	0.0508	23.6	1.9
Whole irises ashed	0.5050	99.0	9.8
Total	0.5558	122.6	11.7
Exp. 2			
Undigested residue	0.2175	22.8	2.4
Combined washings	0.1945	11.1	4.6
Pigment fraction	0.0997	74.5	8.0
Total	0.5117	108.4	15.0
Trypsin blank	0.0534	23.4	2.4
Whole irises ashed	0.4992	79.6	9.8
Total	0.5526	103.0	12.2
Exp. 3 (calves)			
Undigested residue	0.1766	9.0	4.0
Combined washings	0.2048	17.8	4.2
Pigment fraction	0.1207	62.8	5.3
Total	0.5021	89.6	13.5
Trypsin blank	0.0431	12.0	2.8
Whole irises ashed	0.4647	80.0	10.5
Total	0.5078	92.0	13.3

The washings were centrifuged until the supernatant liquid was yellow, and this solution was then poured off. The residue was stirred up with twice-distilled water and

recentrifuged, the operation being repeated five or six times, until no opacity due to protein was observable in the washings. The washings were all added to the original supernatant liquid.

The undigested residue, the washings, the pigment fraction and a blank containing 5 ml. of the trypsin suspension were all dried, ashed and analysed for Zn and Cu by the methods already described. The results are compared in Table 5 with the analysis for the 'control' irises, which had been dried and ashed whole. These results, plus the trypsin blank figures, should equal the total for the various fractions if no contamination or loss has occurred.

Pairs of whale eyes were not available, and digestion of the irises, possibly due to the presence of fat, only partially separated the pigment from the rest of the tissue. Thus it was not possible to construct a balance sheet showing the amounts of Zn and Cu to be found in the pigment fraction and in the other parts of the tissue. However, in the pigment which was separated, Zn and Cu were present in much greater concentration than in the original tissue.

It was likewise found impossible, even after 3 or 4 days digestion, to separate more than a small amount of the pigment from cattle choroids, in the manner used for irises. It was again found, however, that the pigment fraction which was isolated was much richer in Cu and Zn than the original tissue. In Table 6 are collected the results of analyses of dialysed pigment fractions from the various sources mentioned above.

Table 6. *Zinc and copper concentrations in various pigment samples, prepared from eye tissues by the trypsin digestion procedure followed by dialysis*

Source of pigment	Zinc ($\mu\text{g./g. dry material}$)	Copper ($\mu\text{g./g. dry material}$)
Adult cattle irises:		
Batch 1	1000	78
2	730	65
3	719	87
4	748	80
Calf irises	522	44
Humpback whale irises	968	56
Adult cattle choroids	737	48

Further examination of the pigment fraction

Renewed digestion. Part of the pigment fraction obtained from six cattle irises by the above procedure was analysed as such for Zn and Cu, and part was digested with 2% (w/v) trypsin suspension for a further 3 days at 37° before analysis. Results for the two analyses are shown in Table 7.

Table 7. *Zinc and copper concentrations in two pigment preparations before and after a second trypsin digestion*

Pigment preparation	Zinc ($\mu\text{g./g. dry material}$)		Copper ($\mu\text{g./g. dry material}$)	
	Before	After	Before	After
1	871	1073	107	84.9
2	874	1028	113	82.1

Hydrolysis with acid. Part of a pigment fraction was analysed intact, and part weighed and then treated with

0.1N-HCl for 1 hr. on a steam bath. The insoluble residue was centrifuged down, washed, dried, weighed and analysed. Results are shown in Table 8.

Table 8. *Effect of acid hydrolysis upon the zinc and copper concentrations and the dry weight of insoluble material, of an iris pigment fraction*

	Dry weight (g.)	Zinc content ($\mu\text{g.}$)	Copper content ($\mu\text{g.}$)
Original pigment material (A)	0.1225	100	9.1
Insoluble residue after hydrolysis of (A)	0.0741	0.8	8.1

Part of the insoluble residue was retained as a wet suspension. The absorption curves of this suspension in twice-distilled water and of a suspension of the original pigment fraction are given in Fig. 4.

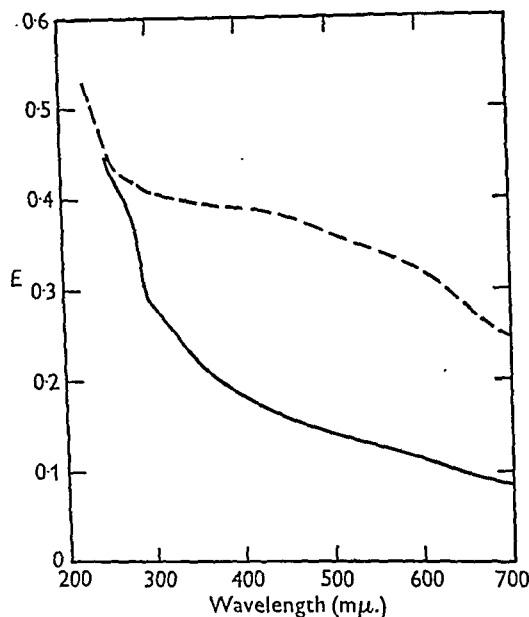


Fig. 4. Absorption spectra of suspensions of melanin material from cattle irises, before and after acid hydrolysis. —, absorption spectrum of the original melanin material suspended in twice-distilled water (pH 5.0). — — —, absorption spectrum of a suspension of the insoluble residue after hydrolysing melanin material.

The amount and nature of the ash of the pigment fraction. The percentage of the dry weight of the pigment fraction recoverable as ash was determined for a sample repeatedly washed and centrifuged down from twice-distilled water, and on a sample dialysed for 3 days against twice-distilled water. Ashing was carried out in a platinum crucible and the respective percentages for the two samples were found to be 2.594 and 2.590.

It is clear that no more than a small part of the ash can be accounted for by Zn and Cu salts or oxides, for these make up only 0.077 and 0.0063%, respectively, of the dry weight of the pigment sample.

It was therefore decided to investigate the other metallic constituents of the ash by a qualitative emission spectroscopic technique.

The Hilger E1-301 version of the Littrow emission spectrograph was used, and an arc source was used for excitation of the electrode and ash.

By means of the Hartmann diaphragm three spectrograms were taken without moving the photographic plate: (a) a control consisting of pure graphite rods; (b) the test material placed on a hollowed graphite cathode opposite a graphite anode and (c) a reference standard consisting of two pure iron electrodes.

Spectrograms were photographed for two wavelength ranges: 2250–2880 and 2880–5050 Å.

The two spectrograms for these ranges, each comprising three spectra in exact juxtaposition, were examined on a Judd Lewis comparator. Lines in the test spectrogram not present in the control were identified by reference to the iron lines in the third spectrogram. The iron spectrogram was charted, and the unknown lines in the test spectrogram identified by reference to labelled photographs in Brode (1939) and the wavelength and element tables in the Massachusetts Institute of Technology *Wavelength Tables* (1939). Unequivocal 'raies ultimes' were identified for Ca, Ba, Mg, Fe, Cu and Zn. The number of iron lines in the test spectrogram was very large and it appears that this element was the major metallic constituent of the ash. These metals, together with Na, were identified in a similar manner in a batch of irises which had been repeatedly washed with twice-distilled water. This demonstrates that they have not been introduced into the material during the digestion or dialysis procedures.

DISCUSSION

The results presented in Table 5 show, without further inquiry, that greater amounts of zinc and copper are associated with the pigment fraction of cattle irises than with any other fraction. However, on close inspection of the results, several matters are seen to require comment.

It was to be expected that the sum of amounts found in the iris fractions would be less than the amount in whole iris since losses due to decomposition of organic compounds are inevitable during digestion and in evaporation at 100° of the large quantities of water present in the washings. Differences between the dry weights and zinc and copper contents of the two irises from the eyes of the same animals are also bound to occur as a result of biological variation and imperfect separation in dissection. Taking these factors into account, there appears to be fairly good agreement between the results for corresponding groups of irises, and it may be concluded that little or no contamination or loss of zinc and copper took place.

The proportion of the total dry weight, zinc content, and copper content, found in each fraction varied somewhat from one experiment to another; this was probably due to variations in the extent of digestion of the tissue and to small variations in the separation technique.

In each experiment, the concentrations of zinc and copper in the dry pigment were much higher than in the other fractions of the original tissue. The accumulation of zinc was much greater than that of copper, and the concentration of zinc was increased by further trypsin digestion of the pigment fraction, whereas that of copper was decreased. Dialysed samples of pigment retained the high concentrations of both metals. Pigment fractions isolated from cattle choroids and from humpback whale irises contained concentrations of zinc and copper similar to those in cattle iris pigment fractions.

Table 5 shows that the high concentrations of zinc and copper in cattle irises can be ascribed to accumulation in the pigment fraction. Table 6 shows that similar accumulations occur in cattle choroids and whale irises. In conjunction with the results in the first part of this paper, it thus appears that the pigment material in mammalian eyes is closely associated with much more zinc and copper than is present in most mammalian tissue constituents.

That the pigment from cattle irises is of the melanin type, is indicated by the retention of colour in the portion of the pigment fraction which is insoluble and resistant to acid hydrolysis. Natural melanins have usually been isolated as the insoluble residues of prolonged acid or alkaline hydrolysis of pigmented tissues. The term 'melanin' is ill-defined; different workers have used different combinations of properties to characterize the material, and it is by no means certain that all the substances which have been given this name have the same chemical structure. The tests for melanin identity employed in the present work are quoted in the classification by Mason (1948).

Physical evidence for the identity of the pigment from cattle irises is given by the absorption curves (shown in Fig. 4) for suspensions of the original pigment fraction, and of the residue, after acid hydrolysis of this fraction. These are similar to those for melanins from other sources (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). Shakir (1948) obtained similar curves for pigment in a supernatant suspension made by grinding choroids; this material had a higher concentration of zinc and copper than the whole tissue. A chemical test used by many workers (e.g. Sachs, 1944) for melanin is the bleaching of the colour by strong oxidizing agents. Concentrated nitric acid rapidly destroyed the colour of the material obtained by us.

A material analogous to the pigment fraction of cattle irises was obtained by Greenstein, Turner & Jenrette (1940), who showed that the black insoluble fraction from mouse melanomas hydrolysed with trypsin contained much protein. This protein

dissolved on acid hydrolysis, leaving a highly coloured residue having a nitrogen content which coincided with that of melanins synthesized from tyrosine.

It is considered that the evidence presented gives sufficient justification for placing the chromogenic material of the pigment fraction from cattle irises in the melanin category.

It has now been fairly well established that mouse-melanoma tissue contains a tyrosinase and that copper is an essential part of this enzyme (Lerner, Fitzpatrick, Summerson & Calkins, 1950). Flesch (1949) suggests that copper becomes bound to the pigment in the neighbourhood of the sulphur atoms of sulphur-containing amino-acids of the attached protein. The relatively large amount of copper in the pigment fraction certainly cannot be due to tyrosinase in the free state, for the enzyme is water-soluble, and would have been removed in the fractionation process.

The results given in Tables 4 and 5 show a difference between the states of zinc and of copper in the pigment fraction. The concentration of copper was slightly reduced by both trypsin digestion and acid hydrolysis. The concentration of zinc was slightly increased by trypsin digestion and reduced almost to zero by acid hydrolysis. This indicates a probable difference between the binding of the two metals in the pigment fraction.

The results of the emission spectroscopic analysis show that the accumulation of metals is not limited to zinc and copper. These two metals together constitute only 0.083 % of the dry weight and 3.21 % of the ash of the pigment fraction. The iron, calcium, magnesium and barium, which were the only other elements identified, together must therefore account for much more of the ash weight than do zinc and copper. The great number and strength of the iron lines in the spectrum indicate that this is a major constituent of the ash.

The demonstration of the presence of barium confirms the report of Ramage & Sheldon (1931) that this metal occurs in irises and choroids of cattle, and in the pigment separated from these tissues by rubbing. They did not find the metal in any other cattle tissues, nor in the choroids of a number of other animals. Waelsch (1932) found that melanin from the choroid gave an ash which was 1.9 % by weight of the dry material; this ash contained a demonstrable concentration of iron.

It thus appears that a number of metals are accumulated in the pigment fraction of mammalian eyes in unusually high concentration. This circumstance may perhaps be connected with a number of other observations. As mentioned in the first part of this paper, white rabbit hair contains much less ash material than black. Gortner (1911) showed that black pigment from rabbit hair, horse hair and

from black feathers contained 2-3 % ash, which was chiefly iron oxide. Rothman & Flesch (1943) isolated a red pigment, containing iron in the ferric form, from bright-red human hair. They stated, however, that such a pigment had not been obtained from any human hair except red, nor from the hair of any other animals. Giuliani (1938) found that dried ink from the squid (*Sepia officinalis*) gave a total ash of 1.86 % of the dry weight, and a copper analysis of 1.17 % of the dry weight.

There exists no direct evidence yet which might settle the problem of whether the high concentrations of these metals in pigmented tissues, and in pigment fractions of these tissues, have any function in developing or maintaining the natural coloration.

SUMMARY

1. Details of modified standard microprocedures for estimating copper and zinc are presented. The methods, as applied to eye tissue, are shown to be specific for these metals.

2. Results are presented for copper and zinc concentrations in eye tissues of sheep, cattle, whales, and rabbits. It is shown that differences in the concentrations of zinc and copper between the various tissues exceed the experimental error.

3. Ranged in respect of zinc and copper concentrations, the tissues fall in roughly the same order in each of the species examined. The highest concentrations, in general, are in the pigmented tissues.

4. The zinc content of the iris or choroid of albino-rabbit eyes is lower than that of the same tissues of pigmented rabbits. There is no corresponding difference in zinc content for the lens or cornea.

5. The zinc and copper of irises occurs mainly in pigment material found in the supernatant liquor from a trypsin digest of the tissue. The concentrations of zinc and copper are higher in the pigment material than the original tissue, and are not reduced by dialysis.

6. The zinc concentration in the insoluble pigment material is slightly increased by prolonged trypsin digestion, but reduced almost to zero by acid hydrolysis. The copper concentration is slightly reduced by trypsin digestion and little affected by acid hydrolysis.

7. The ash of dialysed pigment fractions contains calcium, magnesium, barium and iron, in addition to zinc and copper. Iron appears to be the major constituent.

We are indebted to Dr H. Fore for the benefit of experience gained in a parallel investigation on manganese. The work has been assisted by a grant to the Department and a studentship (J.M.B.) from the Medical Research Council.

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Distribution of Copper and Zinc in the Eyes of Fresh-water Fishes and Frogs. Occurrence of Metals in Melanin Fractions from Eye Tissues

BY J. M. BOWNESS AND R. A. MORTON
Department of Biochemistry, The University of Liverpool

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The results of Leiner & Leiner (1944) showed zinc to be present in the choroid, and other pigmented tissues, of the eyes of fresh-water fishes from Lake Constance, in amounts as much as one hundred times those in similar tissues from mammalian eyes (figures for which are given by Bowness, Morton, Shakir & Stubbs, 1952). Very high concentrations of zinc in the pigmented eye tissues were reported by Leiner & Leiner for each of nine species of fish from Lake Constance; indeed, many were higher than any previously reported to occur normally in living matter (cf. Monier-Williams, 1949). The highest recorded concentration of zinc in animal products, prior to the work of Leiner & Leiner (1944), was for serpent venom, namely 0.56% of the dry weight (Delezenne, 1919). Monier-Williams (1949) attributes even higher concentrations to tench and herrings (Bertrand & Vladesco, 1921). The original paper does not in fact record very high values, and the mistake arose presumably from an erroneous abstract (*Analyst* (1921), 46, 244). The range of zinc concentrations found by Leiner & Leiner in the dry choroids of fresh-water fishes was from 0.44 to 2.96%, and the average for all the species examined was 1.18%.

It was possible that the accumulation of zinc might be a peculiarity arising only in Lake Constance, or alternatively, that very high concentrations of zinc in some eye tissues are common to fresh-water fishes everywhere.

The present investigation had two main objects: (i) to ascertain whether the results obtained by Leiner & Leiner can be confirmed by studies on fishes from Lake Windermere, and (ii) to determine whether the association between zinc concentration and melanin pigmentation found in mammalian eyes (Bowness *et al.* 1952) can be demonstrated for fresh-water fishes. Leiner & Leiner (1944) fractionated a powdered dry homogenate of whole fish eyes by differential centrifugation in an attempt to discover the nature of the material to which most of the zinc was bound. They found that black and grey fractions contained most of the zinc, but, as these accounted for the greater part of the original material, they did not achieve any significant increase in the concentration of zinc, and were unable to indicate the nature of the material to which the metal was bound. In the present work, the trypsin digestion technique (Bowness *et al.* 1952), by which a pigment fraction was separated from mammalian

irises, was found to be effective in separating an analogous black material, rich in zinc, from fish and frog choroids.

MATERIALS AND METHODS

Materials. The heads of trout (*Salmo trutta*, Regan) and whole perch (*Perca fluviatilis*, C.) were obtained through the courtesy of the Freshwater Biological Association at Lake Windermere. The fish were despatched packed in cloth, surrounded by ice in fish tins; the eyes were removed from the heads and dissected at Liverpool within 2 days of the death of the animals. The first batch of perch obtained were young and small, the second batch varied from small to fully grown and aged fish.

Edible frogs (*Rana esculenta*) were obtained alive from Holland through Nederlandsche Heidemaatschappij, Arnhem. The animals were guillotined and the eyes dissected within, at most, a few hours; the retinas were utilized for other work in this Department.

English frogs (*R. temporaria*) were obtained alive from Mr H. F. Ashton of Norwich. The animals were chloroformed, and the eyes dissected, at most within a few hours.

The size of both types of frog varied, but most of them were probably hatched during the previous season.

Dissection of eyes. Certain differences between fish eyes and mammalian eyes should be noted here. In many fish, the inside of the sclera is covered by the silver membrane (argentea). This contains crystals of guanine which produce a silvery-white reflecting surface. In the posterior portion of the eye, between the silver membrane and the choroid, are pockets of blood, which, when the eyes come to be dissected, has clotted. These pockets are associated with the choroid body or 'gland'.

The technique of dissection used was similar to that described by Bowness *et al.* for mammalian eyes. The retinas from fish or frog eyes could be detached without adhering pigment by pulling the optic nerve while exerting a gentle pressure on the eyeball. The retina could then be carefully drawn out of the eye, attached to the optic nerve. With fish eyes, after the separation of the choroid from the blood clot, small pieces of silver membrane were found still to adhere to the tissue; these could only be removed by teasing the choroid with tweezers and a small scalpel. The irises of the fish were never completely separated from argentea.

Analyses for copper and zinc. The eye tissues from the frog *R. temporaria* and from perch were very small; after washing them with twice-distilled water, it was not found practicable to remove the excess water with filter paper. Most of the concentrations are therefore expressed only on a dry-weight basis, wet-weight concentrations having been obtained only for trout and some of the larger tissues of *R. esculenta*.

The analytical method has already been described (Bowness *et al.* 1952). The accuracy of the method as applied to fish-eye tissues has not been assessed experimentally, but it should not be less than that obtained with mammalian material. The ratio of the amount of Zn to a given weight of organic material is much greater for fish eyes than for mammalian eyes; the error in estimating Zn concentration which is due to loss in the course of ashing the organic material is therefore much less important for fish tissues. The generally smaller size of the fish eyes, and the consequent increased greater difficulty of dissection,

resulted, however, in increased uncertainty due to adhering extraneous tissue. It is therefore difficult to assess the extent of uncertainty arising from experimental procedure for the figures given in Tables 1 and 2; in general, it is mainly the third figure which is doubtful.

Separation of pigment fractions. Initially, an attempt was made to separate a pigment fraction from irises, as was done for cattle. However, it was found impossible to remove all the adhering silver membrane. The crystals of guanine accompanied the pigment in the fractionation and could not be removed without the use of hydrolysing agents (e.g. HCl), which are liable to split off the metals from the pigment material.

Attention was then transferred to the choroid. By careful dissection, this could be completely freed from guanine-containing material. It was found to be softer and less fibrous than the choroids of cattle and sheep, and therefore might be expected to be more readily digested by trypsin. Choroid tissue from perch and frogs was, in fact, split up entirely in 3-7 days by digestion with a pancreatin and trypsin suspension, but the process was not successful for trout. At the end of the time required for the choroids to be disintegrated, the digest was filtered through glass wool to remove any undigested tissue, and the filtrate centrifuged until no pigment remained in suspension. The supernatant was poured off, and the black residue stirred up with twice-distilled water and recentrifuged. This procedure was repeated four times. The final residue is the pigment-protein fraction.

Emission spectroscopic technique. Emission spectra of the ash of the perch melanin-protein fraction were recorded by the use of a carbon arc and a high-dispersion Littrow spectrograph (Hilger E. 1-301). Details of the technique used for identifying the elements present are given by Bowness *et al.* (1952).

RESULTS

The main findings are displayed in Tables 1 and 2. The recorded results have been calculated directly from the experimental readings.

In order to test whether high concentrations of zinc, such as those in some of the eye tissues, are of general occurrence in the body of the perch, analyses were carried out on liver, muscle and skin.

Proportion of the copper and zinc in the perch choroid bound to the insoluble pigment-protein fraction. The dry matter in 73.3 mg. of fresh perch choroid was found to weigh 8.6 mg. When 309.2 mg. of fresh choroids, corresponding to 36.2 mg. dry weight, were digested with trypsin and a pigment-protein fraction isolated from the supernatant, the dry weight of this fraction was 14.9 mg. The weights of zinc and copper present in these amounts of choroid and pigment-protein material can be calculated by using the appropriate concentrations of the metals given in Tables 1 and 2. The ratio of the weight of zinc or copper in 14.9 mg. pigment-protein to that in 36.2 mg. choroid shows the proportion of the total weight of these metals in the choroid which is in non-ionic combination with the pigment-protein material. For zinc, this proportion

Table 1. *The concentrations of copper in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations in $\mu\text{g./g.}$ dry tissue; the figures in brackets underneath (when given) show the concentration in $\mu\text{g./g.}$ wet tissues corresponding to the figures immediately above. A dash means that the tissue was absent, or not available for analysis.)

Tissue	Trout (fresh-water)	Perch	Frog	
			<i>R. temporaria</i>	<i>R. esculenta</i>
Lens	5.62 (2.42)	0.85	29.8	27.4 (13.6)
Cornea	18.4 (3.5)	21.0	104	—
Sclera	8.65 (2.05)	27.3	120	199 (8.87)
Retina	35.8 (4.85)	18.1	117	—
Argentea	199.0	17.1	—	—
Iris plus ciliary body	105.0 (26.5)	18.8	—	—
Choroid	87.8 (18.0)	30.5	133	—
Pigment-protein fraction of choroids	—	49.3	—	985

Table 2. *The concentrations of zinc in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations expressed as $\mu\text{g./g.}$ dry tissue; the figures in brackets underneath, when given, show the concentrations in $\mu\text{g./g.}$ wet tissue corresponding to the figures immediately above.)

Tissue	Trout (fresh-water)	Perch	Frog	
			<i>R. temporaria</i>	<i>R. esculenta</i>
Lens	15.7 (6.75)	15.6	78.5	16.3 (8.1)
Cornea	81.3 (11.5)	189	278	—
Sclera	457 (95.6)	343	326	266 (132)
Retina	580 (74.2)	728	500	—
Argentea	7020	2010	—	—
Iris plus ciliary body	5990 (1560)	6380	—	—
Choroid	15100 (3100)	23800	14400	—
Pigment-protein fraction of choroids	—	43800	—	28100

is $\frac{43800}{23800} \times \frac{14.9}{36.2} \times 100 = 74.7\%$ (see Table 2); for copper, $\frac{49.3}{30.5} \times \frac{14.9}{36.2} \times 100 = 53.0\%$ (see Table 1).

The pigment-protein material, to which these fractions of the total copper or zinc are bound, constitutes 41.6% of the dry weight, or 4.82% of the wet weight of the choroid.

Table 3. *Concentrations of zinc in perch skin, muscle and liver*

Tissue	Zinc concentration ($\mu\text{g./g.}$ dry tissue)
Skin (white)	31.0
Muscle	57.7
Liver	59.6

Absorption spectra of pigments. Light passing through a suspension of particles in water is partly scattered and partly absorbed by the particles. The scattering of light by a particle depends on its size. The absorption curve of a suspension will, therefore, be governed by two factors, the light absorption of the substance suspended (itself dependent on the structure of the compound), and the size and number of particles suspended.

The absorption curves of suspensions in water of melanin-protein fractions from perch and frogs are given in Fig. 1.

Hydrolysis of pigment-protein fraction. Prolonged trypsin digestion of melanin-protein fractions from perch and frogs was found gradually to lower their zinc and copper contents. The zinc content on dry

weight of the perch material was reduced from 4.38 to 4.1% by 3 days' incubation with trypsin suspension. It is possible, therefore, that the original figure of 4.38% is too low by virtue of the incubation with trypsin during the separation procedure.

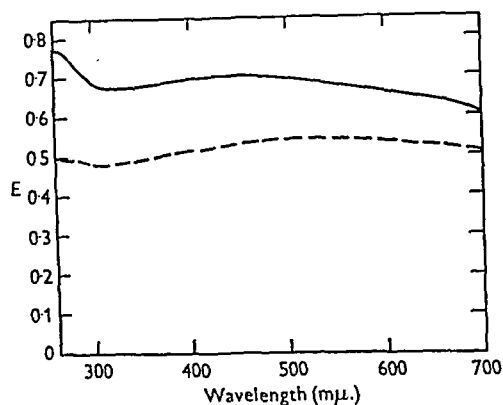


Fig. 1. Absorption spectra of melanin fractions from fish and frog eyes. —, perch pigment; ---, frog (*R. esculenta*) pigment.

Acid hydrolysis of the pigment was found to have an effect analogous to that obtained with cattle pigment. Part of the dry melanin-protein fraction (8.3 mg.) from perch was treated with 10 ml. 0.1N- H_2SO_4 for 1 hr. at 100°. The resulting mixture was centrifuged, the residue being washed with twice-distilled water and recentrifuged twice. The final residue was dried, weighed, ashed and analysed for zinc.

choroid will include much dialysable material, so that there is evidently a higher concentration of bound metals in the pigment-protein fraction than in the original choroid.

Emission spectroscopic analysis of the ash from perch material showed that the main metallic constituents of the pigment-protein fraction are zinc, calcium, and iron. Minor constituents were aluminium, copper, magnesium and strontium. No trace was found of barium, which was identified in the ash of cattle pigment-protein fraction.

DISCUSSION

It is evident from Table 4 that the high concentrations of zinc obtained by Leiner & Leiner (1944) are not due to Lake Constance as a special habitat.

In all seven of the other species of fish from Lake Constance examined by Leiner & Leiner, the iris, choroid, and silver membrane had the highest zinc contents of any of the eye tissues. Of these three, the choroid usually had the highest concentration, but in the Burbot the iris was higher in zinc; in no case was the silver membrane highest.

In comparing the results for fish from Lake Constance and Lake Windermere there are several points to note. Leiner & Leiner give the name of their trout as 'Sea-trout' (*Trutta lacustris* L.). There is some dispute as to whether the trout all belong to the same genus, but in the present work the view of Regan (1911), who classified all the English trout as *Salmo trutta*, is followed. There must remain some doubt, however, as to whether both sets of fish are of the same species. Secondly, in considering the

Table 4. A comparison of the results of Leiner & Leiner (1944) for perch and trout eye tissues with those obtained in the present work

Tissue	Trout ($\mu g.$ Zn/g. dry tissue)		Perch ($\mu g.$ Zn/g. dry tissue)	
	Leiner & Leiner	Present work	Leiner & Leiner	Present work
Lens	16.0	15.7	20	15.6
Retina	540-990	580	1090-1780	728
Iris plus ciliary body	6960-8700	5990	6200-7710	6380
Silver membrane	6360	7020	8200	2010
Choroid body	980-1490	—	510-1730	—
Choroid	9200-11350	15100	24000-29600	23800

The dry weight of the residue was 4.1 mg. and the zinc content 2.4 $\mu g.$ The estimated zinc content of the original melanin-protein fraction would be 365.2 $\mu g.$

Acid hydrolysis has thus removed nearly all the zinc and half the dry weight of the original material.

Ash content of the melanin-protein fraction. The amount of dry material remaining as ash after incineration was found to be just over 10% for the whole choroid, and just under 10% for the pigment-protein fraction from perch. The ash from the

silver membrane, it should be noted that the tissue is very small and it is not possible to separate it entirely from pigmented material. The amount of pigment remaining in attachment is partly a matter of chance, and there is therefore much latitude for variation in the results. Thirdly, the choroid body was not analysed in the present work because it appeared to be mainly composed of blood; it was completely dissected away from the choroid and silver membrane and thus does not enter into any of the present results.

The zinc concentrations in the eye tissues of the frog fairly closely parallel those of the fresh-water fish. Leiner & Leiner (1942) studied the concentrations of zinc in the eye tissues of many salt-water Teleosts. The general range of zinc concentrations was lower than that in fresh-water fish and frogs, but higher than that in mammals; for example, the range of zinc concentrations for the dry choroids of all the species of salt-water fish studied was 530–9800 $\mu\text{g./g.}$ (Leiner & Leiner, 1944). In all the fish examined, whatever the absolute amounts of zinc present, the same tissues contain the highest concentrations of zinc. Zinc concentrations in the eye tissues decrease in the following order: choroid or iris, retina, sclera, cornea, lens. The argentea concentrations are rather variable. Excluding this last-named tissue, which does not occur in mammals, and excluding the mammalian lens, the same order is true for results obtained on cattle, sheep, whale and rabbit eyes (Bowness *et al.* 1952). The range, from highest to lowest zinc concentration, is very much smaller in mammals than in fish. The concentration in the lens of mammalian eyes is generally slightly higher than that of fish eyes, yet the concentration in the choroid may be only 1/100th that of the same fish tissue.

Table 3 shows that, despite the high concentrations of zinc in the eye tissues, other parts of the perch have much the same concentrations as those given by Lutz (1926) for various mammalian species.

The results for copper concentrations in various eye tissues do not follow any clear-cut general pattern. The variation in the order of eye tissues, arranged according to copper concentrations, is much greater than is the case for zinc. The physico-chemical and biochemical agencies which accumulate zinc appear, in all the vertebrate eyes examined, to be active in one tissue more than another, and most active in the same tissues in all species. The same cannot be true for copper. In general, pigmented tissues and pigment-protein fractions from all the vertebrate eyes examined by us contain relatively high copper concentrations. In some species, other tissues may have concentrations as high, or higher. In trout, the silver membrane has a higher copper content than either choroid or iris. In connexion with this, it was observed that the debris from an attempted digestion of mixed choroids and silver membranes of trout, which consisted largely of guanine, had a high copper content (672 $\mu\text{g./g.}$ dry tissue). Frog-eye tissues seem to have a relatively high copper content, the pigment-protein fraction of *R. esculenta* being especially high in copper.

The very high concentrations of zinc in the pigment-protein fractions of perch and frogs, and the observation that at least 74% of all the zinc in

perch choroids is bound in non-ionic form to this fraction, show that the high zinc contents of the pigmented eye tissues can largely be accounted for by an association with melanin pigmentation.

As compared with up to 2.6% found in material from cattle, the ash content of dry perch pigment-protein fraction was about 10%, of which calcium and iron were shown to be fairly abundant constituents. Zinc, however, constituted nearly half of this ash, as compared with only 3.21% of the cattle material. Iron appeared to be the major metallic constituent of the cattle pigment-protein ash, and barium, which was not found in the perch material, was present as a minor constituent. It seems, therefore, that the absolute amount of the metallic elements associated with the pigment-protein material, and the relative proportions of each, vary from one vertebrate species to another.

The absorption curves of pigment-protein fractions from perch and frog eyes differ from those obtained from cattle (Bowness *et al.* 1952) and from those of other melanins (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). The ratio of the light absorption in the ultraviolet region to that at higher wavelengths is much lower for the fish material than for the others. This may be caused partly by the different size of the particles in suspension, but such a large difference probably also indicates a difference in chemical structure.

Though it appears established that there is a connexion between melanin pigmentation and the presence of high concentrations of zinc (and possibly other metals) in the pigmented eye tissues, and perhaps in other pigmented tissues (cf. Bowness *et al.* 1952), the function of such metallic accumulations remains an open question. Leiner & Leiner (1941) showed that the zinc contents of the tissues of fish and mammals could not be accounted for solely by the amount of metal bound to carbonic anhydrase. They showed that zinc could lessen the inhibition of carbonic anhydrase by various tissue constituents. There seems no reason, however, why such a function should require a great accumulation of zinc in the pigmented eye tissues of fish, and especially in fresh-water fishes.

As stated previously (Bowness *et al.* 1952), the accumulation of copper in pigment fractions cannot be explained by the presence of free tyrosinase. The only other organic copper compounds known to occur in vertebrates are haemocuprein and hepatocuprein (Keilin & Mann, 1938), but these also are relatively water-soluble and could not account for the copper in the pigment fraction unless they were combined in some insoluble form.

Possible functions of the metals associated with the melanin-protein fractions of pigmented tissues will be discussed in a further paper.

SUMMARY

1. Results for the copper and zinc contents of eye tissues of perch, trout, and frogs are presented. The results of Leiner & Leiner (1944) for zinc in perch and trout are confirmed.

2. Most of the zinc, and half the copper, of the perch choroids is bound in non-ionic form to the pigment-protein fraction of the tissue.

3. The absorption spectra of pigment-protein suspensions from perch and frog eyes differ from those of cattle eyes.

4. On incineration, the perch pigment-fraction was found to leave about 10 % of the dry weight as ash. Calcium, iron and strontium were constituents of this ash, but almost half is zinc (calculated as metal).

5. The association between zinc and melanin pigmentation is discussed.

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Effects of High-voltage Cathode Rays on Aqueous Solutions of Tryptophan, Tyrosine, Phenylalanine and Cystine

BY B. E. PROCTOR AND D. S. BHATIA*

Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

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The effects of irradiation by high-voltage cathode rays on amino-acids in fish muscle and on aqueous solutions of histidine monohydrochloride have already been reported (Proctor & Bhatia, 1950; Bhatia & Proctor, 1951). The irradiation caused no significant destruction of any one of ten amino-acids in fish. It was shown, however, that histidine in aqueous solution is decomposed by irradiation with cathode rays through deamination and fission of the glyoxaline ring.

Stenström & Lohmann (1928) concluded from their experiments on X-ray irradiation of aqueous solutions of tyrosine, that the phenolic structure of this amino-acid is changed by the irradiation. They also irradiated solutions of cystine and noticed no measurable change when the colorimetric method of Folin & Looney (1922) was used to determine cystine. Stenström & Lohmann (1931) also re-

ported that ammonia was given off from tyrosine and that tryptophan was destroyed upon irradiation with roentgen radiations. Buckhman & Manoilov (1949) found that cysteine evolves hydrogen sulphide on irradiation with ultraviolet rays. Dale & Davies (1951) studied the liberation of hydrogen sulphide by X-radiation from aqueous solutions of cysteine and glutathione.

The present study was undertaken to ascertain the effects of various doses of cathode rays on aqueous solutions of DL-tryptophan, DL-phenylalanine, L-tyrosine and L-cystine in various concentrations.

EXPERIMENTAL

Stock solutions of the amino-acids were prepared in concentrations of 10 mg./ml. A weighed quantity of the amino-acid was dissolved in a minimum quantity of 20% (w/v) HCl, and the solution was made to volume with distilled water. From these stock solutions, test solutions were made as desired.

* Present address: Central Food Technology Research Institute, Mysore, India.

Table 1. *Effect of high-voltage cathode rays on aqueous solutions of amino-acids*

Amino-acid and dose of cathode rays (r.e.p.)	Percentage retention of amino-acid in solutions.			
	Amino-acid concn. ($\mu\text{g./ml.}$)			
	100	250	500	1000
L-Cystine				
Control	100.0	100.0	100.0	100.0
100 000	45.8	72.2	92.0	97.7
250 000	38.2	66.6	79.9	95.0
500 000	22.7	53.7	70.0	90.2
1 000 000	0.0	35.7	61.3	82.0
DL-Phenylalanine				
Control	100.0	100.0	100.0	100.0
100 000	56.5	76.8	88.1	91.6
250 000	23.6	57.4	75.6	86.4
500 000	3.1	33.1	57.9	79.6
1 000 000	0.0	9.6	35.3	70.8
L-Tyrosine				
Control	100.0	100.0	100.0	100.0
100 000	50.1	77.6	79.3	88.4
200 000	42.3	69.3	74.1	88.3
400 000	27.9	55.5	71.4	83.9
800 000	6.9	34.1	59.4	76.6
DL-Tryptophan				
Control	100.0	100.0	100.0	100.0
100 000	46.4	81.0	92.2	94.8
250 000	43.0	68.6	91.2	97.0
500 000	30.0	58.8	77.6	90.0
1 000 000	10.0	36.6	62.2	79.4

Table 2. *Ionic yields of the decomposition, by high-voltage cathode rays, of amino-acids in aqueous solutions*

Amino-acid and dose (r.e.p.)	Ionic yields* in solutions.			
	Amino-acid concn. ($\mu\text{g./ml.}$)			
	100	250	500	1000
L-Cystine				
100 000	0.85	1.08	0.62	0.36
250 000	0.39	0.52	0.62	0.31
500 000	0.24	0.36	0.47	0.31
1 000 000	—	0.25	0.30	0.28
DL-Phenylalanine				
100 000	0.99	1.32	1.35	1.90
250 000	0.69	0.97	1.10	1.24
500 000	0.44	0.76	0.96	0.93
1 000 000	—	0.51	0.74	0.66
L-Tyrosine				
100 000	1.03	1.16	2.77	2.40
200 000	0.60	0.80	1.35	1.20
400 000	0.38	0.56	0.74	0.84
800 000	0.24	0.43	0.53	0.60
DL-Tryptophan				
100 000	0.49	0.35	0.36	0.48
250 000	0.21	0.23	0.14	0.11
500 000	0.13	0.15	0.21	0.19
1 000 000	0.08	0.12	0.17	0.19

* Ionic yield = molecules of amino-acid destroyed per ion pair; 1 r.e.p. = 1.6×10^{12} ion pairs/g.

A pressure-insulated Van de Graaff electrostatic generator (Trump & Van de Graaff, 1948) operating at 3 000 000 V. was used for the cathode-ray irradiation, the technique for irradiation being the same as that previously described (Bhatia & Proctor, 1951). Each amino-acid was irradiated in four concentrations at four dose levels.

Microbiological determinations of the amino-acids were made with *Leuconostoc mesenteroides* P.60 as the test organism. For the estimation of cystine, tryptophan, and phenylalanine, the basal medium of Dunn, Shankman, Camien, Frankl & Rockland (1944) was used, and for tyrosine, the basal medium developed by Steele, Sauberlich, Reynolds & Baumann (1949).

RESULTS

No colour change was observed in the irradiated solutions of cystine. A dull-yellow pigmentation was produced in the irradiated solutions of tryptophan, phenylalanine and tyrosine. The intensity of the colour increased progressively with dose at a given concentration and also with concentration at a given dose of irradiation. When the irradiated solutions were allowed to stand overnight in the refrigerator, the intensity of the colour did not change except with tryptophan, where the pigmentation became more intense on standing.

The characteristic odour of hydrogen sulphide was noticed in all the irradiated solutions of cystine. The presence of hydrogen sulphide was confirmed by the lead acetate test. The intensity of the dark spot on the lead acetate paper, inserted into the bottle of irradiated solution, increased progressively with cathode-ray dose at a given concentration. No attempt was made to determine the evolution of hydrogen sulphide quantitatively.

Table 3. *Decomposition of DL-phenylalanine by high-voltage cathode rays*

(*G* value = number of molecules reacting/100 eV. (electron volts) of energy.)

Concentration of solution, <i>C</i> (g./ml.)	Inactivation dose, <i>D</i> (r.e.p.)	Specific-inactivation dose <i>D/C</i> (r.e.p./g./ml.)	Average <i>G</i> value
1.0×10^{-4}	1.64×10^5	1.64×10^9	4.26
2.5×10^{-4}	4.27×10^5	1.70×10^9	4.09
5.0×10^{-4}	8.89×10^5	1.78×10^9	3.93

The percentage retention of the amino-acids in the aqueous solutions after irradiation is shown in Table 1. The cathode-ray doses are expressed in terms of roentgen-equivalent-physical (r.e.p.), as described by Evans (1947) 1 r.e.p. = 83 ergs/g.

The ionic yields of the decomposition of amino-acids by cathode rays (calculated from the data in Table 1) are shown in Table 2.

From the data in Table 1, calculations have been made of the inactivation dose, the specific-inactivation dose, and the *G* values (*G* value = number of molecules reacting/100 eV. of energy) (Table 3) for

phenylalanine at three concentrations. The pH values of all solutions before irradiation are given in Table 4.

Table 4. *pH of solutions of amino-acids prior to irradiation*

Amino-acid	Amino-acid concn. (μg./ml.)			
	100	250	500	1000
L-Cystine	2.90	2.59	2.39	2.14
DL-Phenylalanine	2.84	2.50	2.25	2.00
L-Tyrosine	2.61	2.31	2.05	1.80
DL-Tryptophan*	2.78	2.44	2.21	—

* At a concentration of 50 μg./ml. the pH was 2.94.

DISCUSSION

All the amino-acids were decomposed upon irradiation, and the decomposition was related exponentially to the dose. The difference in the amino-acid content of the solution before and after irradiation is considered as the amount decomposed. The dilute solutions were relatively more affected by a given dose than were the more concentrated solutions.

The ionic yields of the decomposition of L-cystine over a range of concentrations of from 100 to 1000 μg./ml. were (with a single exception) less than 1.00. The ionic yields of the decomposition of DL-tryptophan over a range of concentrations of from 50 to 500 μg./ml. were less than 1.00 in all instances. For tyrosine, ionic yields greater than 1.00 were obtained at all concentrations when the irradiation dose was 100 000 r.e.p., and at concentrations of 500 and 1000 μg./ml. when the dose was 200 000 r.e.p. The ionic yields in the more dilute solutions were less than 1.00. The ionic yields of the decomposition of phenylalanine were less than 1.00 except in a few solutions containing 250, 500, and 1000 μg. of the amino-acid/ml.

It will be noted that the ionic yields, as measured microbiologically by inactivation of cystine, is of the order 0.26–0.36 for a solution with a concentration of 1000 μg./ml. This agrees with the value of 0.2 found by Dale & Davies (1951) for a cysteine solution of the same concentration (based on hydrogen sulphide production). The reason that Dale & Davies found no liberation of hydrogen sulphide from cystine may be because the irradiation doses they used were much lower than those used in the present investigation.

The inactivation dose for phenylalanine was found to be dependent on its concentration in solution over a range of concentrations of from 100 to 500 μg./ml., but the specific inactivation doses were constant. Under the experimental conditions studied, the *G* values were of the order of four

molecules reacting/100 eV., which agrees with the values given by Allen (1947) for organic liquids.

These observations are all indicative of an indirect action of high-voltage cathode rays on these amino-acids. For ionic yields higher than 1.00, the same explanation as given previously for histidine (Bhatia & Proctor, 1951) would probably be valid, namely, that molecular fragments of the amino-acids resulting from cathode-ray treatment competed for the free radicals.

Evolution of hydrogen sulphide from cystine indicates that the molecule of cystine is probably decomposed at the disulphide linkage.

SUMMARY

1. Aqueous solutions of L-cystine, DL-phenylalanine, L-tyrosine, and DL-tryptophan in various concentrations were irradiated with high-voltage cathode rays at doses ranging from 10^5 to 10^6 r.e.p. Determinations of the amino-acid contents of the

solutions were made microbiologically after irradiation.

2. All four amino-acids were decomposed upon irradiation, and the decomposition was related exponentially to the dose.

3. Ionic yields were determined for the four amino-acids over a wide range of concentrations.

4. Data are presented to show that the action of high-voltage cathode rays on these amino-acids took place through free radicals being intermediates.

5. Evolution of hydrogen sulphide from the cystine solution indicated that cystine was probably decomposed at the disulphide linkage upon irradiation with high-voltage cathode rays.

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Some Chemical Properties of Helvolic Acid

By T. I. WILLIAMS*

Sir William Dunn School of Pathology, University of Oxford

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Burton & Abraham (1952) reported that cephalosporin P_1 , an antibiotic produced by a species of *Cephalosporium*, resembled helvolic acid (Chain, Florey, Jennings & Williams, 1943). Further work by Burton, Cardwell & Abraham (1951) has confirmed this resemblance and has allowed a simple interpretation of the results of a chemical investigation into helvolic acid, completed by the author in 1945 and described in this paper.

The isolation of helvolic acid from culture filtrates of *Aspergillus fumigatus* mut. *helvola* Yuill has been

described by Chain *et al.* (1943). Helvolic acid was shown to be a colourless crystalline compound, melting point 212° . It is readily soluble in most organic solvents, except light petroleum, but is insoluble in water; its sodium salt is readily soluble in water. Helvolic acid was assigned the formula $C_{32}H_{44}O_8$. It titrated as a monobasic acid and a monomethyl ester was prepared by the action of diazomethane.

These preliminary observations on the chemical properties of helvolic acid were confirmed and extended in the investigation now described, which was directed primarily to the discovery of the role of the oxygen atoms in the molecule.

* Present address: 'Endeavour', 26 Dover Street, London, W. 1.

The helvolic acid required in the present investigation was produced by the method previously described (Chain *et al.* 1943). From a total of 5200 l. of crude culture filtrate, worked up in twenty-seven batches, 5.5 g. of pure helvolic acid were obtained. Attempts to increase this yield by varying the culture medium and the conditions of growth were unsuccessful.

An investigation of the rate of development of anti-bacterial activity, assayed by the ordinary plate-and-cylinder method used for penicillin, showed that when the mould was grown as a surface culture at 25°, in a synthetic medium of glucose and mineral salts, a peak activity was reached after about 8 days' growth. Thereafter, the activity fell considerably to a minimum at 16–18 days. This was followed by a second increase to a fairly constant value at 21 days, at which time the crop was harvested for the isolation of helvolic acid: it was found that the antibacterial activity of cultures of 6–8 days' growth was due, not to helvolic acid, but to gliotoxin. An account of this work has been published elsewhere (Glister & Williams, 1944).

Waksman, Horning & Spencer (1943) described an antibiotic, which they named fumigacin, produced by *A. fumigatus* Fres. Menzel, Wintersteiner & Hoogerheide (1944) showed that fumigacin was a mixture of gliotoxin and helvolic acid; their account of helvolic acid, as far as it goes, agrees in almost all essentials with the findings of the present author.

A point of discrepancy, however, is the value of the specific rotation. Chain *et al.* (1943) gave the value $[\alpha]_D^{20} = -49.4^\circ$ in chloroform; Menzel *et al.* gave $[\alpha]_D^{25} = -132^\circ \pm 2^\circ$. Two separate, highly purified, samples have now been found to give $[\alpha]_D^{23} = -113^\circ$ (c, 3.1) and -117° (c, 2.6) respectively. The difference still existing may be due to contamination with gliotoxin of the material used by these workers; gliotoxin is strongly laevorotatory ($[\alpha]_D^{25} = -254^\circ$). The possibility of contamination was raised by Menzel *et al.* and not entirely disproved. Their product reduced Fehling's solution slowly at 100°. Our purified samples have no action on Fehling's solution even on heating for 1 hr. at this temperature. Gliotoxin, however, readily reduces Fehling's solution.

RESULTS

Nature of oxygen atoms. All the analytical and degradative evidence supports the formula $C_{32}H_{42-44}O_8$. For helvolic acid, Menzel *et al.* proposed a formula $C_{29}H_{38-40}O_7$; several objections to this will be discussed later.

The absence of enolic or phenolic properties in helvolic acid show that the acidic properties are due to a carboxyl group. This is confirmed by the preparation of a monomethyl ester (m.p. 262°) by the action of diazomethane on the acid. Analysis of the

ester gave results in conformity with a formula $C_{33}H_{44-46}O_8$. The molecular weight was found by an X-ray crystallographic method (Crowfoot & Low, 1943) to be 556 ± 14 ($C_{33}H_{46}O_8$ requires 570).

A monosemicarbazone and a dioxime of helvolic acid, and a monosemicarbazone of its methyl ester, have been prepared. The absence of reducing properties and the positive Zimmermann reaction indicate that at least one of the carbonyl groups is ketonic. Menzel *et al.*, using a different preparative method, obtained a monoxime.

An acetoxyl determination by the standard (acid hydrolysis) method of Elek & Harte (1936) gave values of 8.3 and 7.8% acetoxyl for the free acid and 7.9% for the methyl ester. Menzel *et al.* record similar results for this method. The calculated values, assuming the formula $C_{32}H_{44}O_8$ for the acid, are 7.74 and 7.54% respectively for one acetoxyl group. The volatile acid was identified as acetic acid. Drs Weiler and Strauss (Dyson Perrins Laboratory, Oxford) carried out an acetoxyl determination on helvolic acid by hydrolysing it for 15 min. at 100° with 0.1N-ethanolic sodium hydroxide. After acidification, the volatile acids were removed by steam distillation and collected in excess of standard alkali. They obtained values of 16.3 and 15.6% acetoxyl, i.e. roughly twice those given by the method of Elek & Harte. These results suggest that, while helvolic acid contains two acetoxyl groups, one is much more readily split off than the other. Since helvolic acid is virtually insoluble in acid solution, the conditions of the Elek & Harte method of estimation do not favour the removal of an unreactive acetoxyl group.

If helvolic acid is hydrolysed in 0.1N-alkali for 36–48 hr. at 37°, approximately one equivalent of alkali is taken up, in addition to that required to neutralize the carboxyl group, showing the liberation of a second acidic grouping. This probably corresponds to hydrolysis of the more reactive of the two acetoxyl groups. A crystalline sodium salt of the new acid is precipitated during hydrolysis. The corresponding acid has been obtained in a crystalline form. Its equivalent weight by direct titration was 590; on again treating with excess alkali at 37°, its equivalent was very nearly half this, namely 298. Further hydrolysis with alkali gave, after acidification, a substance which no longer dissolved readily in alkali and which analysed for $C_{28}H_{36}O_5$. This suggests that hydrolysis of the second acetoxyl group gives an hydroxyl so situated that it readily lactonizes with the free carboxyl group (Burton *et al.* 1951).

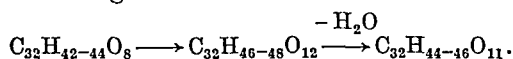
On the evidence adduced above, all the eight oxygen atoms known to be present in the helvolic acid molecule may be satisfactorily accounted for. Two occur in a carboxyl group, two in carbonyl groups, and the remaining four are distributed

between two acetoxyl groups, one of which is much more resistant than the other to acid hydrolysis. The hydroxyl group liberated on hydrolysis of this second acetoxyl group is so situated that it readily lactonizes with the carboxyl group.

Reaction with bromine. Helvolic acid behaves as an unsaturated substance, decolorizing bromine water and permanganate in the cold and reacting readily with ozone. When the acid is treated in the cold with excess bromine in glacial acetic acid, four atoms of bromine react per molecule of acid, but the low bromine content of the product indicates that the main reaction here is oxidative.

Oxidation with hydrogen peroxide. Helvolic acid is readily and smoothly oxidized by hydrogen peroxide (perhydrol) or by periodic acid, to the same neutral compound, m.p. 229–230°, the properties of which are consistent with a formula $C_{32}H_{46}O_{11}$.

One possible explanation is that this compound results from the formation of a diglycol at the site of two double bonds, followed by loss of water to form a lactone ring.



On prolonged hydrolysis at 37° with 0.1N-alkali, the compound slowly dissolves; its equivalent weight under these conditions is 160. The molecular weight corresponding to $C_{32}H_{46}O_{11}$ is 606 and thus four equivalents of alkali are apparently involved in the alkaline hydrolysis of the perhydrol oxidation product; this has been characterized as a dioxime (additional evidence for the presence of two carbonyl groups in helvolic acid itself) and as the benzoyl and 3:5-dinitrobenzoyl esters. With ketene, a monoacetyl derivative was obtained. In view of the evidence that helvolic acid itself contains two acetoxyl groups, it seems that the two remaining hydroxyl groups believed to be present in the perhydrol product are, therefore, rather inert.

Further oxidation with lead tetraacetate. Recognition of the presence of two double bonds led to attempts to split up the molecule by attacking it at these points. With cold alkaline permanganate an immediate reaction takes place, involving ten atoms of oxygen per molecule; there is a further slow reaction in which more than twenty oxygen atoms per molecule are involved. Oxidation with chromium trioxide appears to follow a similar course. It was not possible, however, to characterize any degradation products. With lead tetraacetate, a neutral crystalline derivative was obtained; melting point above 200° (decomp.). Analysis indicated a formula $C_{32}H_{44}O_{11}$; the molecular weight by X-ray crystallography (Crowfoot & Rogers, 1944) is 595 ± 15 ($C_{32}H_{44}O_{11}$ requires 604).

Helvolic acid and its methyl ester react readily with ozone. The acid itself appears to yield two

acidic fractions; the methyl ester yields a neutral product. The products could not, however, be characterized.

Helvolic acid is rapidly oxidized by selenium dioxide in ethanol at 100°, but again it was not possible to isolate any degradation product.

The possibility of dehydrogenation with selenium was explored, but preliminary experiments, and the experience of other workers with different substances, indicate that this method is not likely to be fruitful unless the oxygen content of the molecule can first be reduced.

Reduction. Helvolic acid is very resistant to reduction; after attempted reduction with phosphorus and iodine (6 hr. at 100°) unchanged acid was recovered in good yield.

DISCUSSION

So far as common ground is covered, the results described here are, in almost all respects, in agreement with those of Menzel *et al.* (1944). On questions of fact they differ significantly only in the finding that helvolic acid has no reducing properties. The evidence is entirely consistent with a formula $C_{32}H_{42-44}O_8$ for helvolic acid. Menzel *et al.* are inclined to favour a formula $C_{29}H_{38-40}O_7$, but there are several objections to this. In particular, it corresponds to a molecular and equivalent-weight (498 or 500) well outside the limits (562 ± 12) set by the X-ray crystallographers (Crowfoot & Low, 1943). The American workers themselves record equivalent weights of 514, 560 and 591; their molecular weights were determined by the Rast method, which is very much less reliable than X-ray crystallography. A further objection to the C_{29} formula is that we have found good evidence for the existence of eight oxygen atoms in the molecule. The analytical results obtained by Menzel *et al.* agree almost equally well with either formula, but for the silver salt they record values practically identical with the theoretical values for $C_{32}H_{43}O_8Ag$.

The eight oxygen atoms required by the formula $C_{32}H_{44}O_8$ can be satisfactorily accounted for as follows: two in a carboxyl group; four in acetoxyl groups; two in carbonyl groups (at least one ketonic). Helvolic acid behaves as an unsaturated compound, and the course of the reaction with perhydrol, as well as other evidence, suggests that two double bonds are present. The fact that the perhydrol oxidation product is a neutral compound, and that there is evidence that it contains a lactone ring, makes it probable that one double bond is in a position γ or δ to the carboxyl group.

No direct evidence has been obtained about the kind of carbon skeleton present in the molecule. Dr R. E. Richards, of the Physical Chemical Laboratory, Oxford, investigated the infrared

*Reaction of helvolic acid with perhydrol
and with periodic acid*

Oxidation proceeds smoothly, giving the same product in either case. The following procedures are typical:

(1) Helvolic acid (65.9 mg.) was dissolved in 7 ml. of glacial acetic acid and 1 ml. perhydrol added. The mixture was allowed to stand for 16 hr. at room temperature and then evaporated to dryness in an evacuated desiccator. The residue was crystallized from 70% acetic acid. Yield 28.8 mg.

(2) The reagent was a 2.5% solution of sodium periodate in 75% acetic acid. Helvolic acid (23.6 mg.) was dissolved in 2.5 ml. of reagent and solution allowed to stand for 40 hr. at 37°. The mixture was diluted with 5 ml. of water and the precipitate dissolved in CHCl_3 . The CHCl_3 layer was separated, washed and evaporated at room temperature. The residue was recrystallized from 70% acetic acid. Yield 10 mg.

The identity of the product obtained by the two methods was proved by analysis, identity of melting points, and mixed, melting point. It was finally proved (Crowfoot & Rogers, 1944) by the complete identity of the X-ray crystallographic pictures. The product is neutral, crystallizing from acetic acid as small, colourless needles, m.p. 229–230°. It is insoluble in water. (Found: C, 64.4, 63.0, 64.4; H, 7.6, 7.6, 8.0; mol.wt. (Rast), 560. $\text{C}_{32}\text{H}_{46}\text{O}_{11}$ requires C, 63.4; H, 7.6; mol.wt. 606.) The perhydrol product dissolves slowly in 0.1 N-NaOH. 15.5 mg., treated for 7 days at 37° with 0.1 N-NaOH, went completely into solution. The quantity of alkali used up corresponded to an equivalent of 160.

A dioxime was prepared by the method used for helvolic acid. (Found: N, 4.3. $\text{C}_{32}\text{H}_{46}\text{O}_{11}\text{N}_2$ requires N, 4.4%.)

By reaction with excess ketene in ethereal solution at room temperature, a crystalline monoacetyl derivative was obtained; m.p. above 225° (decomp.). (Found: for two specimens C, 64.8, 64.2; H, 7.6, 7.6; Ac, 22.3. $\text{C}_{34}\text{H}_{48}\text{O}_{12}$ requires C, 64.0; H, 7.4; Ac, 19.8%.) By reaction with benzoyl chloride for 16 hr. in pyridine solution at room temperature, a crystalline monobenzoyl derivative was obtained, m.p. 248°. (Found: C, 66.8; H, 6.4. $\text{C}_{38}\text{H}_{50}\text{O}_{12}$ requires C, 65.9; H, 7.0%.)

A 3:5-dinitrobenzoyl derivative was prepared in the same way, m.p. above 215° (decomp.). (Found: N, 3.6. $\text{C}_{39}\text{H}_{48}\text{O}_{16}\text{N}_2$ requires N, 3.5%.)

Further oxidation of perhydrol oxidation product with lead

tetraacetate. The reagent used was a saturated solution of lead tetraacetate in glacial acetic acid. Helvolic acid (92.9 mg.) was converted to perhydrol oxidation product, and the latter was dissolved in 5 ml. of the tetraacetate reagent. After standing at 37° for 16 hr. the mixture was diluted with 5 vol. distilled water and shaken for 3 hr. to decompose excess tetraacetate. The solution was extracted with 5 ml. CHCl_3 and the CHCl_3 extract evaporated to dryness. The residue (72.8 mg.) was crystallized twice from hot ethanol. A neutral product was obtained, crystallizing in short needles, melting point indeterminate (decomp.). (Found: for two specimens C, 62.1, 63.5; H, 6.8, 7.2. $\text{C}_{32}\text{H}_{44}\text{O}_{11}$ requires C, 63.6; H, 7.3%.) Molecular weight was found by X-ray crystallography (Crowfoot & Rogers, 1944) to be 595 ± 15 ($\text{C}_{32}\text{H}_{44}\text{O}_{11}$ requires mol.wt., 604).

SUMMARY

1. All analytical and degradative evidence suggests that helvolic acid has the formula $\text{C}_{32}\text{H}_{42-44}\text{O}_8$. It titrates as a monobasic carboxylic acid.

2. The following derivatives of helvolic acid have been prepared and characterized: the semicarbazone, dioxime, methyl ester, silver salt, and a product obtained by oxidation with either perhydrol or periodic acid. The latter appears to be a lactone.

3. From the methyl ester, a semicarbazone has been prepared. From the perhydrol oxidation product, a dioxime, an acetoxy, a benzoyl, and a 3:5-dinitrobenzoyl derivative have been prepared.

4. The behaviour of helvolic acid and certain of its derivatives on alkaline hydrolysis has been studied. Results indicate the presence of two acetoxy groups in the original molecule.

5. Helvolic acid behaves as an unsaturated substance containing two double bonds.

It is a pleasure to record my thanks to Dr E. Chain, F.R.S., now of the Istituto Superiore di Sanità, Rome, for his encouragement and advice while the original experimental work was being done.

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lated by multiplying the total nitrogen by the factor 6.38. *Lactose* was determined polarimetrically by Vieth's method as described by Elsdon & Walker (1942), the necessary correction being made for the volume of the precipitate. *Chloride* was estimated by the method of Davies (1938). The *freezing point* was estimated by the improved Hortvet apparatus described by Temple (1937). *Calcium, magnesium and sodium* were estimated in milk samples which were evaporated in vitreosil basins on a water bath prior to being ashed in an electric muffle furnace which was thermostatically controlled at 600°. The ash was dissolved in warm 6*N*-HCl and made up to a known volume. The final stage in the estimation of calcium was the titration of the precipitated calcium oxalate with KMnO₄ as described by Hawk, Oser & Summerson (1947). Sodium was precipitated as sodium zinc uranyl acetate in sintered-glass filters, dried at 100° and weighed (Peters & Van Slyke, 1932). Magnesium was determined from the phosphate content (Fiske & Subbarow, 1925) of a magnesium ammonium phosphate precipitate. *Potassium* was precipitated as cobaltinitrite from a 2% trichloroacetic acid (TCA) filtrate of whole milk and the cobalt in the centrifuged and washed precipitate was determined by its colour reaction with nitroso-R-salt (Sideris, 1942). The potassium figures were corrected for the volume of the protein precipitated by TCA (Rowland, 1938) and for co-precipitated sodium. *Creatinine* was determined by treatment of the tungstic acid filtrate of milk with alkaline picrate solution, the resulting colour being read in a Spekker absorptiometer. *Creatine* was determined by subtracting the preformed creatinine from the total creatinine determined by the same method after autoclaving the protein-free filtrate with HCl (Hawk *et al.* 1947). *Phosphatase* was determined as already described by Chanda & Owen (1951). *Ascorbic acid* was estimated according to the method used by Mattick *et al.* (1945), precaution being taken that the samples of milk were made large enough to fill the preserving jars in which they were taken so as to prevent any aerobic oxidation. The milk was pipetted into the TCA-metaphosphoric acid mixture within 15 min. of the collection of the samples, to avoid formation of any dehydroascorbic acid. *Riboflavin* was determined in a Spekker fluorimeter by the method of Emmerie (1938) combined with Arnold's (1945) modification. *Aneurin* was determined fluorimetrically in isobutanol as thiochrome. The aneurin was partitioned as described by Houston, Kon & Thompson (1940). Free aneurin was determined in skimmed milk by Jansen's (1936) method, while cocarboxylase was determined from the difference between free aneurin and aneurin present in the takadiastase digest of the TCA extract of skim milk. Pepsin digestion of the TCA precipitate gave the protein-bound aneurin. Total aneurin (free plus cocarboxylase plus protein-bound) was independently determined by digestion of skim milk with takadiastase only, for it was found in confirmation of Houston *et al.* (1940) that pepsin digestion following takadiastase digestion did not materially increase the total.

RESULTS AND DISCUSSION

Milk yield. Typical graphs showing the increases in milk yield brought about by thyroxine and the decreases brought about by thiouracil have already been given (Chanda & Owen, 1951). The quantitative responses in milk yield produced by the two drugs in

all the cows used in the present series of experiments are now described. In calculating the positive or negative responses in milk yield it is obviously difficult to estimate what the yield would have been had no treatment been given, since after the first few weeks from calving, the yield of milk gradually declines. The increases caused by thyroxine and decreases caused by thiouracil were therefore complicated by this natural decline. To offset this difficulty, the average daily yield (x) of each cow during period 1 was analysed by co-variance with the average daily yield (y) in period 2, the cows being divided into three treatment groups—control, thyroxine and thiouracil. From the total sum of squares and products the variance due to treatment was eliminated. From the residual sum of squares and products, the following significant regression equation was established between x and y

$$y = -0.44 + 0.94077x$$

(x = the actual milk yield in lb. in period 1; y = the expected milk yield in period 2). Geometrically, this method of statistical analysis corresponds to using an extrapolation of a straight line fitted to the milk yield data of period 1 as the expected yield in period 2 (Owen, Smith & Wright, 1943). Since the effects of discontinuance of the hormone treatment are just as marked as those produced by its institution, period 3 cannot be regarded simply as a second control period, so that a line fitted to the data of periods 1 and 3 would overestimate the effect of

Table 1. *Effect of thyroxine and thiouracil on milk yield*

Group of cows	No. of cows	Expected milk yield during period 2 calculated from the regression (lb./day)*	Actual milk yield (lb./day)	Response (%)
Control	9	21.1	21.1	—
Thyroxine (10 mg./day)	9	17.1	19.3	+13.2
Thiouracil (20 mg./day)	8	22.0	19.6	-10.7

* 1 lb. = 454 g.

thyroxine and underestimate that of thiouracil. The expected milk yields during period 2, calculated from this equation, compared favourably with the actual milk yields shown simultaneously by each of the nine control cows. The results for all the nine thyroxine-treated cows except one showed that the actual yields in period 2 were higher than the expected yields. For the sake of brevity the mean results of each group are recorded in Table 1 which shows that the average response was +13.2% in the thyroxine-treated cows during treatment, while

the average response was -10.7% in the eight cows treated with 20 mg. thiouracil daily. The response by the cow in Exp. 1, which received only 10 mg. thiouracil, was only -5.8% . The responses in the individual cows within groups varied very widely and the initial yields of cows did not seem to have any effect on the relative magnitude of the response caused by either thyroxine or thiouracil.

Fat, solids-not-fat and protein content of milk. The results for solids-not-fat have been calculated to a fat-free basis. In most of the animals, thyroxine caused an increase in the fat content of the milk. The effect of the hormone on the solids-not-fat content of milk was variable. In some of the cows there was a small increase, but in others there was no change. This contrast is particularly noticeable in cow no. 12 (Exp. 3) in which the greatest response in the fat content of milk was recorded without any change in the solids-not-fat. Thiouracil decreased the fat content of milk except in cow no. 8 (Exp. 2) for which the fat content of milk was considerably increased in successive periods. The effect of thiouracil on the solids-not-fat content of the milk was inconstant.

had the opposite effect. An increase in chloride content with a corresponding decrease in lactose was also noticeable in the control cows with the progress of lactation. There was thus a close negative correlation between the average lactose and chloride contents in Table 3 ($r = -0.9933$). Davies (1936) pointed out that since lactose and chloride account for 80% of the osmotic pressure of milk, there tends to be a constant relationship between these two constituents. He found from analysis of numerous milk samples that the relationship can be expressed as

$$\text{lactose content} = 6.26 - 13.5 \times \text{chloride content},$$

or in other words lactose + 13.5 times chloride should be constant and equal to 6.26. In the present investigation this constant has been calculated and recorded in Table 3 in which it is referred to as the lactose-chloride number. It was found that this number was fairly constant for all the cows in all the periods, but its actual value was somewhat lower than that of Davies (1936). The mean value in the present experiments was found to be 6.19. In conformity with this constancy of the relationship

Table 2. *Effect of thyroxine and thiouracil on the contents of fat and solids-not-fat in milk*

Exp.	Cow	Daily injection in period 2	Fat (g./100 g. milk)			Solids-not-fat (g./100 g. fat-free milk)		
			Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
1	1	None	4.07	3.75	4.15	8.78	8.89	9.15
	2	10 mg. thyroxine	3.83	4.20	5.00	8.84	9.11	9.92
	3	10 mg. thiouracil	3.82	3.23	3.38	8.46	8.21	8.57
2	4	None	4.45	4.34	4.78	8.86	8.87	9.20
	5	10 mg. thyroxine	4.51	5.08	4.83	9.45	9.96	9.61
	6	10 mg. thyroxine	4.31	4.43	4.61	8.75	8.63	8.97
	7	20 mg. thiouracil	4.06	3.62	3.46	8.48	8.53	8.52
	8	20 mg. thiouracil	4.19	4.66	5.13	8.79	8.83	9.33
3	9	None	3.95	3.90	4.24	8.92	8.89	8.78
	10	None	4.28	3.95	3.98	8.88	8.81	8.54
	11	10 mg. thyroxine	4.12	4.74	4.35	9.24	9.43	8.95
	12	10 mg. thyroxine	4.14	5.24	4.74	9.01	8.97	8.72
	13	20 mg. thiouracil	4.44	4.12	4.58	9.05	8.85	8.76
	14	20 mg. thiouracil	3.91	3.47	4.32	9.02	9.01	8.84

The protein content of the fat-free milk in the cows used in Exps. 2 and 3 confirmed the findings in Exp. 1 already reported by Chanda & Owen (1951). Neither thyroxine nor thiouracil had any effect on the protein content of the fat-free milk.

Lactose, chloride and freezing point. These analyses, which were carried out in Exps. 1 and 2 only, show that, corresponding to a small increase in solids-not-fat (Table 2), there was a small increase in the lactose content of the milk, but the effect was not noticeable in all the treated cows. Thiouracil caused a small decrease in lactose content corresponding to a small decrease in solids-not-fat. Simultaneously with an increase in lactose content, there was a decrease in the chloride content of the milk in the thyroxine-treated cows. Thiouracil

between lactose and chloride it was also found that the freezing point of the milk remained unchanged throughout the experiments (Table 3).

Calcium, magnesium, sodium and potassium. The calcium content of the milk showed no change attributable to hormonal effects, thus confirming the earlier observations of Owen (1948b). In conformity with this finding is the observation that calcium was not affected by thiouracil. In Exp. 3, the mean calcium contents, with their standard errors, of the two thyroxine-treated cows were 136.7 ± 3.1 , 130.8 ± 3.1 and 121.2 ± 3.0 mg./100 g. milk in periods 1, 2 and 3 respectively. The corresponding figures for the two cows which were treated with thiouracil were 124.4 ± 2.8 , 118.8 ± 3.0 and 118.3 ± 2.8 , and for the two control cows 121.2 ± 3.3 ,

114.8 ± 3.4 and 110.3 ± 3.1 . The failure of hormonal treatments to change the calcium content of milk is in sharp contrast to their effects on the phosphorus content which was increased by thyroxine (Owen, 1948*b*; Chanda & Owen, 1951) and decreased by thiouracil (Chanda & Owen, 1951). As pointed out earlier (Owen, 1948*b*), this can perhaps be related to the small amount of calcium required by the soft tissues. These observations, in conjunction with that of Owen (1948*b*), indicate that the metabolic pathways of calcium and phosphorus in adult animals are not so directly linked in the soft tissues as they are known to be in bone.

There was no indication that either drug caused any change in sodium, potassium or magnesium. The means and standard errors for all animals, without distinction of periods, were 64.3 ± 4.8 , 149.6 ± 3.6 and 11.3 ± 0.4 mg./100 ml. milk for sodium, potassium and magnesium respectively.

experiment was, however, by injection, whereas, in the treatment of toxic goitre in humans, thiouracil is given by mouth and much larger doses of the drug per unit body weight are used. Since leucopaenia caused by thiouracil is an idiosyncrasy it is conceivable that treatment of a larger number might demonstrate its occurrence in cows. We have, however, never observed any untoward symptoms in cows treated with thiouracil. The average figures for blood constituents of all the cows in Exp. 3 were: haemoglobin 8.80 ± 0.17 g./100 ml., red blood cells 5.11 ± 0.14 millions/mm.³, and white blood cells 9.31 ± 0.42 thousands/mm.³.

Creatine and creatinine content of milk. To find whether the increase in ester phosphorus caused by thyroxine (Chanda & Owen, 1951) could be attributed to an increase in labile phosphate, such as creatine phosphate, the creatine content of the milk was estimated in Exp. 3. The results showed that

Table 3. *Effect of thyroxine and thiouracil on the lactose and chloride contents of milk*

Exp.	Cow	Daily injection in period 2	Period	Lactose (%)	Chloride (%)	Lactose chloride number*	Depression of freezing point (° C.)
1	1	None	1	4.13	0.151	6.17	0.545
			2	4.09	0.155	6.18	0.541
			3	4.05	0.158	6.18	0.542
	2	10 mg. thyroxine	1	4.22	0.145	6.18	0.546
			2	4.47	0.125	6.16	0.539
			3	4.16	0.152	6.21	0.544
	3	10 mg. thiouracil	1	4.33	0.137	6.18	0.538
			2	4.06	0.156	6.17	0.542
			3	4.37	0.139	6.25	0.546
2	4	None	1	4.39	0.132	6.17	0.542
			2	4.21	0.148	6.21	0.539
			3	4.09	0.155	6.18	0.541
	5	10 mg. thyroxine	1	4.67	0.112	6.18	0.545
			2	5.18	0.076	6.21	0.546
			3	4.76	0.105	6.18	0.542
	6	10 mg. thyroxine	1	4.28	0.144	6.22	0.539
			2	4.32	0.138	6.18	0.542
			3	4.11	0.154	6.19	0.537
	7	20 mg. thiouracil	1	4.25	0.142	6.17	0.547
			2	4.29	0.140	6.18	0.541
			3	4.35	0.139	6.23	0.545
	8	20 mg. thiouracil	1	4.31	0.138	6.17	0.551
			2	4.01	0.159	6.16	0.547
			3	4.44	0.135	6.26	0.549

* Lactose content + $13.5 \times$ chloride content = 6.26 (Davies, 1936).

Blood analysis. In view of reports that thiouracil treatment of toxic goitre in humans causes leucopaenia in a minority of patients (Grainger, Gregson & Pemberton, 1945; Himsworth, 1948), the blood of the cows in Exp. 3 was analysed for haemoglobin, red blood cells and white blood cells. The daily variations could not be related to the drug treatments, and there was no evidence of leucopaenia in the cows. The method of treatment in the present

neither thyroxine nor thiouracil caused any change in the content of either creatine or creatinine. An analysis of variance showed that there was no significant variation in the creatine content of the milk between animals. There was, however, a significant decrease from period to period. This variation was attributable to advancing lactation since there was a regular trend of decrease during successive periods in all the cows. The averages

in periods 1-3 of creatine+creatinine (measured as creatinine) were 13.7 ± 0.50 , 13.1 ± 0.52 and 11.5 ± 0.62 mg./100 g. in the thyroxine-treated cows, respectively. The corresponding figures for the cows which received thiouracil were 14.0 ± 0.49 , 13.2 ± 0.54 and 11.4 ± 0.62 , and for the control cows 12.8 ± 0.50 , 13.4 ± 0.50 and 11.8 ± 0.54 mg./100 g. Creatine contributed a relatively constant percentage to these totals ($71.2 \pm 4.1\%$).

The lack of change in the creatine content of milk in thyroxine-treated cows is in sharp contrast to that of urinary creatine which was found to be markedly increased in cows treated with thyroxine (Owen, 1948a). The statement of Basu & Mukherjee (1943) that ester phosphorus in milk is exclusively the labile creatine phosphate is not supported by these results. In spite of the large increase in the ester phosphorus caused by thyroxine (Chanda & Owen, 1951), no changes occurred in the creatine content of the milk. Moreover, the amount of creatine found in the milk could have accounted for only 1.4 mg. ester phosphorus, which is lower than the smallest ester phosphorus figure observed by Chanda & Owen (1951) during thiouracil treatment.

Water-soluble vitamins

Ascorbic acid. The results for the five cows in Exp. 2 are recorded in Fig. 1 which shows that, in the thyroxine-treated cows, the ascorbic acid content of the milk decreased during treatment, while in the cows which were treated with thiouracil it increased. The control cow showed that the stage of lactation had little effect on the ascorbic acid content of the milk. In Exps. 1-3 the mean decrease caused by 10 mg. thyroxine in five cows was 25% (range 14-34%) while the mean increase caused in four cows by 20 mg. thiouracil was 24%. In the cow dosed with 10 mg. thiouracil the decrease was 10%.

Decreases in ascorbic acid content of milk after treatment of cows with iodinated casein have been recorded by Van Landingham, Henderson & Weakley (1944). Similar decreases were observed by Bartlett, Rowland & Thompson (1949) who attributed them to the iodine in the iodinated casein. Since, however, iodinated casein was not used in the present experiments, it is clear that it is the thyroxine itself which produces these effects. The minute dose of iodine given in 10 mg. thyroxine per day could not be expected to influence the ascorbic acid content of the milk. Furthermore, there is other evidence, albeit indirect, that the thyroid probably governs the ascorbic acid content of milk.

Riboflavin. The mean riboflavin contents of the milk of six cows (Exp. 3) in individual periods are recorded in Table 4 with their standard errors. The

mean results for the two thyroxine-treated cows showed a decrease in the treatment period (period 2) compared with the initial control period (period 1), but this also occurred in one of the control cows and in the two thiouracil cows. The results were therefore analysed statistically. The analysis of variance, also recorded in Table 4, showed that the differences between cows were significant, but that the differences between periods within cows were not. The mean values in period 1 showed a range of 83-127 μ g./100 ml. fat-free milk. It can, therefore, be concluded that neither the drugs nor the stage of lactation caused any change in the riboflavin content.

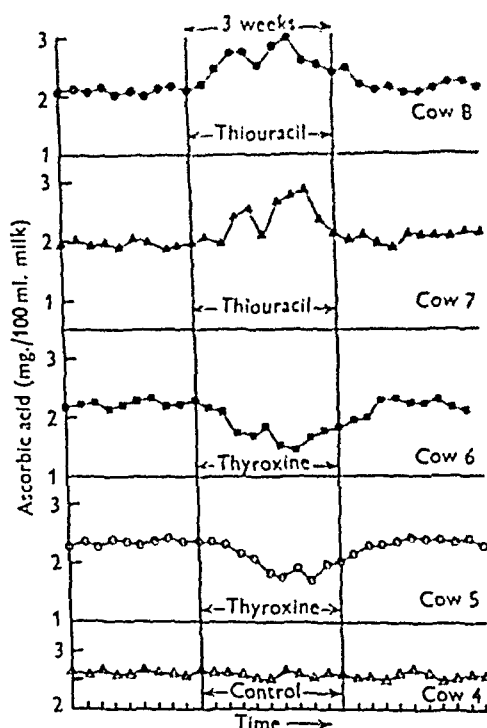


Fig. 1. Effect of thyroxine and thiouracil on the ascorbic acid content of cow's milk.

Bartlett *et al.* (1949) reported a 17% decrease in riboflavin in the milk of cows treated with thyroxine. The Arizona workers (Kemmerer, Bolomey, Vavich & Davis, 1946) also reported a decrease, the concentration of riboflavin during the treatment period dropping almost to nil. These values, as Kon & Henry (1949) pointed out, are inconceivably low. In view of the finding of Bartlett *et al.* (1949) the present results of 2-day values were further analysed statistically to compare the rates of decrease in groups of cows during period 2. The differences between the three regressions were found not to be statistically significant. Kon & Henry (1949), quoting Thompson (1945), stated that neither thyroxine nor iodinated protein had any effect on the riboflavin content of milk.

Table 4. *Effect of thyroxine and thiouracil on the riboflavin content of cow's milk*

Cow	Daily injection in period 2	Riboflavin ($\mu\text{g./100 ml. fat-free milk} \pm \text{s.e.}$)		
		Period 1	Period 2	Period 3
9	None	103.2 \pm 1.6	105.4 \pm 2.2	102.2 \pm 4.2
10	None	82.5 \pm 1.7	77.2 \pm 2.9	77.0 \pm 2.4
11	10 mg. thyroxine	111.5 \pm 2.8	97.5 \pm 3.1	108.1 \pm 3.6
12	10 mg. thyroxine	103.0 \pm 1.5	95.1 \pm 2.9	100.2 \pm 3.4
13	20 mg. thiouracil	104.6 \pm 1.6	103.5 \pm 2.6	99.5 \pm 4.1
14	20 mg. thiouracil	127.2 \pm 2.2	115.7 \pm 2.5	107.0 \pm 4.2

Analysis of variance of the average results for riboflavin

(N.S., not significant.)				
Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (e^2)
Total	17	2687.58	—	—
Cows	5	2302.18	460.44	20.39*
Periods	2	159.61	79.81	3.53 (N.S.)
Error	10	225.79	22.58	—

* $P < 0.001$.

Total aneurin and the partition of aneurin. The cocarboxylase and protein-bound aneurin in the milk studied in Exp. 3 are shown graphically in

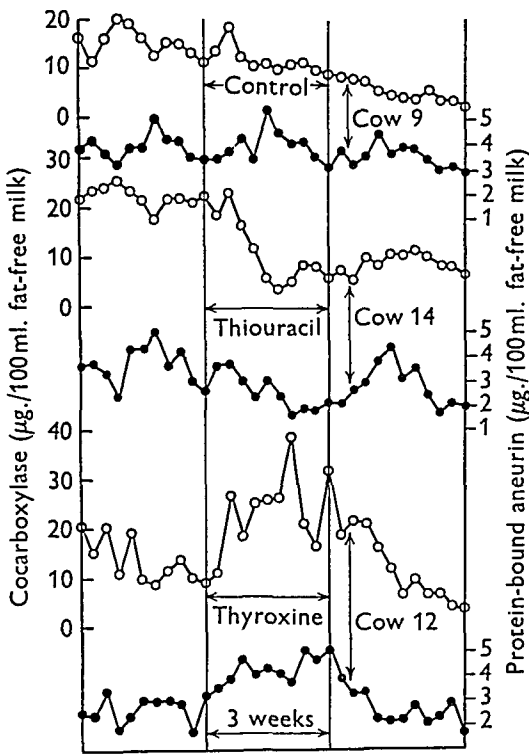


Fig. 2. Effect of thyroxine and thiouracil on the cocarboxylase and protein-bound aneurin content of cow's milk. ○—○, cocarboxylase; ●—●, protein-bound aneurin.

Fig. 2. In this diagram the individual 2-day data throughout the experiment are shown. The average values for total aneurin in each period are recorded

in Table 5, where the phosphatase and the partition of aneurin between the free, the cocarboxylase and the protein-bound forms are also recorded. It can be seen from Table 5 that the total aneurin content of the milk gradually decreased in all the cows in successive periods. There were large variations in the total aneurin values between cows. An analysis of variance of the total aneurin showed that the differences between cows and between periods were statistically significant. Due to the significant difference between cows, comparisons cannot be made between the controls and the treated cows in the same period; and from a comparison of the same cow from period to period, the effect of the hormone treatment is difficult to establish, but it would appear that the normal rate of the decrease was slightly retarded by thyroxine and somewhat enhanced by thiouracil. In spite of the smallness of the change in the total aneurin, the partition of aneurin showed considerable change. Table 5 shows that under the influence of thyroxine the cocarboxylase in the milk increased markedly at the expense of the free aneurin. There was also a small increase in the protein-bound aneurin. These changes are well shown in Fig. 2. Decreases in the free form of aneurin in the milk of cows fed iodinated protein were reported earlier by Bartlett *et al.* (1949). The present experiments have shown that these decreases are accompanied by an increase in the cocarboxylase and in the protein-bound forms of aneurin when thyroxine is administered subcutaneously. At the peak of hormonal response, the cocarboxylase in the thyroxine cow no. 12, accounted for 38 % of the total aneurin compared with only 21 % in the pretreatment period. The present results showed that thiouracil increased the free aneurin content and decreased the contents of both

Table 5. Partition of aneurin in fat-free milk

Cow	Daily injection in period 2	Period	No. of analyses	Total aneurin (µg./100 ml.)	Percentage of total aneurin as			Phos- phatase (phenol units)*
					Free vitamin	Cocarbony- lase	Protein- bound	
9	None	1	11	39.4	50.4	38.0	7.3	59
		2	10	37.0	55.6	31.0	7.0	58
		3	11	37.1	72.7	14.9	6.9	109
10	None	1	11	46.5	48.0	39.9	7.6	47
		2	10	41.6	64.3	24.8	6.5	67
		3	11	40.5	70.6	18.5	6.6	78
11	10 mg. thyroxine	1	11	46.5	61.2	26.2	5.9	70
		2	10	46.0	30.7	58.2	7.3	35
		3	11	41.1	62.2	26.2	6.9	84
12	10 mg. thyroxine	1	11	42.0	58.1	31.5	6.7	67
		2	10	41.0	28.4	58.1	10.8	21
		3	11	39.2	56.9	30.9	7.0	73
13	20 mg. thiouracil	1	11	45.8	45.0	42.5	7.6	39
		2	10	39.1	68.4	21.1	7.3	70
		3	11	37.1	67.2	21.6	7.4	73
14	20 mg. thiouracil	1	11	50.3	42.7	44.6	7.4	37
		2	10	45.0	66.8	23.4	5.7	61
		3	11	44.3	68.3	20.5	6.4	59

* See Chanda & Owen (1951).

coccarboxylase and protein-bound aneurin (Fig. 2). The same sort of effect was noticed in the control cows (Table 5) as in the thiouracil cows, but it is evident from Fig. 2 and Table 5 that these natural effects were intensified by thiouracil. Thus, the minimum percentage of total aneurin in the form of coccarboxylase was decreased from 10 % in period 1, to 8 % in period 2 in the control cow (no. 10) while the corresponding decrease was from 18 to 4 % in the thiouracil-treated cow (no. 14).

.During the last period of the experiment, the combined effect of advancing lactation and the cessation of thyroxine treatment produced some dramatic changes. The phosphatase titre increased rapidly and there was a big increase in free aneurin at the expense of coccarboxylase (Table 5). Protein-bound aneurin was also reduced. When thiouracil was discontinued, advancing lactation offset the expected sharp decrease in the phosphatase titre (Table 5) but protein-bound aneurin and coccarboxylase increased (Fig. 2). The effect of advancing lactation can be seen in the control animal (no. 9), in which, during period 3, a marked increase in phosphatase was accompanied by an increase in free aneurin and by corresponding decreases in coccarboxylase and protein-bound aneurin (Table 5). These effects were as unmistakable as those produced artificially by thiouracil in cows 13 and 14.

Correlation between phosphatase and phosphorylated aneurin. A positive correlation between phosphatase and free aneurin was observed to be a normal occurrence in the milk of cows and goats (Houston *et al.* 1940) and of sows (Braude *et al.* 1947) and also in human milk (Chanda, Owen & Cramond, 1951). This was confirmed in the present work.

There was a close positive correlation between phosphatase and free aneurin in the milk of all the cows (Table 6), and these correlations held whether the results for free aneurin were expressed in units per 100 ml. milk or as percentages of the total

Table 6. Correlations of the percentages of aneurin in the free and coccarboxylase forms with phosphatase in the cow's milk

(Number of pairs of observations was 28 in each case.)

Cow	Treatment	Coefficient* of correlation of phosphatase with	
		Free aneurin × 100	Coccarboxylase × 100
		Total aneurin	Total aneurin
9	None	+0.9490	-0.8960
10	None	+0.8932	-0.8473
11	Thyroxine	+0.8794	-0.8898
12	Thyroxine	+0.9514	-0.9185
13	Thiouracil	+0.8419	-0.8257
14	Thiouracil	+0.8328	-0.7869

* Every coefficient shown in the table is significant at $P < 0.001$.

aneurin. There were also (Figs. 3 and 4) large negative correlations (Table 6) between phosphatase and coccarboxylase. The curves in Figs. 3 and 4 fitted by the method of least squares, show for a thyroxine and a thiouracil cow a curvilinear relationship between phosphatase and coccarboxylase similar to that found in human milk by Chanda *et al.* (1951).

In Fig. 5, in which mean values for phosphatase and for protein-bound aneurin are plotted, the regression line, fitted by the method of the least squares indicates a significant negative but non-linear correlation between the two variables.

Biochemical significance of the relation of phosphatase to phosphorylated aneurin. Like ester phosphorus, which was increased dramatically by thyroxine (Chanda & Owen, 1951), cocarboxylase and protein-bound aneurin were also markedly increased by the hormone. Thiouracil caused a significant decrease in cocarboxylase. Normally a higher proportion of phosphorylated aneurin is a characteristic of early lactation (Houston *et al.* 1940;

increase. The larger amount of phosphorylated aneurin appeared to occur at the expense of free aneurin because the total aneurin remained approximately constant. With thiouracil the proportion of free aneurin was increased at the expense of the phosphorylated fractions. When the mammary gland worked faster under the influence of thyroxine more of the phosphoric esters were synthesized (Chanda & Owen, 1951). This in turn was accompanied by an increase in the cocarboxylase. During

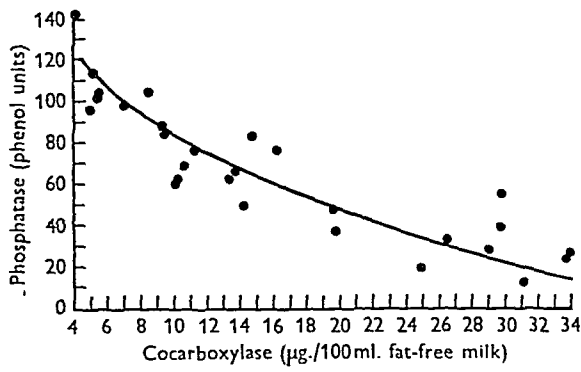


Fig. 3. Correlation between cocarboxylase and phosphatase in the milk of thyroxine-treated cow no. 11.

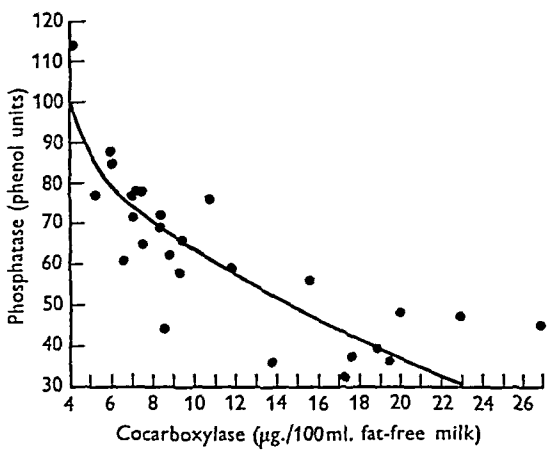


Fig. 4. Correlation between cocarboxylase and phosphatase in the milk of a thiouracil-treated cow.

Chanda, 1951). In late lactation the amount of phosphorylated aneurin is only 10% of the total aneurin so that the ratio of total to free aneurin approaches unity. The higher proportion of phosphorylated aneurin in early lactation and during thyroxine treatment may be related to the greater activity of the mammary gland. The phosphatase titre in milk becomes small, perhaps due to its retention in the gland at the peak of lactation or when thyroxine is administered to the cow. As the free aneurin in the milk of thyroxine-treated cows was observed to decrease with a concomitant decrease in the phosphatase, the contents of cocarboxylase and protein-bound aneurin were found to

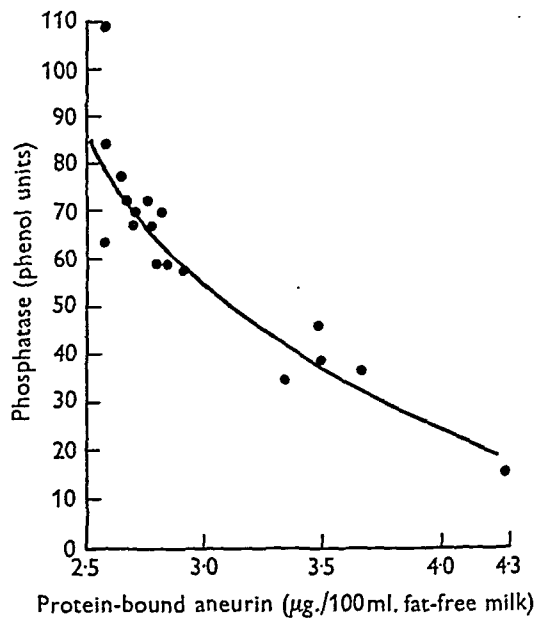


Fig. 5. Correlation between protein-bound aneurin and phosphatase in cow's milk. The ordinate of each point represents the mean value of phosphatase for a given cow in a given period, and its abscissa represents the corresponding mean value of protein-bound aneurin.

hypothyroidism there is a lowering in the metabolic rate and consequently less of the milk aneurin is in the phosphorylated form. The lesser amount of phosphatase secreted in the milk during thyroxine treatment (Folley & White, 1936) may therefore denote a retention of the enzyme for synthetic activity in the gland. This hypothesis is supported by the close negative correlations observed between phosphatase and phosphorylated aneurin. The function of the milk phosphatase may thus be anabolic in the mammary tissue even though in milk after secretion it appears to be mainly hydrolytic (Graham & Kay, 1934). The interdependence of phosphorylated compounds and phosphatase may be related to the power of thyroxine to increase the concentration of enzymes in the body (Williams-Ashman, 1948). The reverse changes caused by thiouracil are also explicable on this basis. The negative correlations between phosphorylated compounds and phosphatase observed in this and in

previous experiments (Chanda *et al.* 1951; Chanda & Owen, 1951) may imply a synthetic role for phosphatase in syntheses by the mammary gland.

SUMMARY

1. The effect of thyroxine and thiouracil on the composition of cow's milk has been investigated, and the relation of phosphatase to the partition of aneurin has been specially studied. The following are the main results:

2. Thiouracil (20 mg. per cow per day subcutaneously) decreased the milk yield of cows by 10.7 %, while DL-thyroxine (10 mg. per cow per day subcutaneously) increased it by 13.2 %.

3. Thyroxine caused an increase in the fat of milk in some of the cows and thiouracil caused a decrease.

4. There was a small increase in the lactose content of milk in the thyroxine-treated cows corresponding to a simultaneous decrease in the chloride content. Thiouracil caused the reverse effects. There was an inverse relationship between lactose and chloride, such that

$$\text{lactose content} = 6.19 - 13.5 \times \text{chloride content.}$$

The freezing point of the milk was not changed by either drug.

5. The calcium, sodium, potassium, magnesium, protein, creatine and riboflavin contents of the milk were not demonstrably affected by either thyroxine or thiouracil. The creatine content of milk decreased with advancing lactation. Creatine phosphate was not found in the milk.

6. Thyroxine decreased the ascorbic acid content of the milk by 25 %, while thiouracil caused an increase of 24 %. When the dosage of thiouracil was halved, the increase was only 10 %.

7. Thyroxine did not affect the total aneurin content of the milk, but thiouracil caused a small but statistically significant decrease. Thyroxine increased markedly the cocarboxylase content of the milk, while a small but statistically significant increase also occurred in the protein-bound aneurin fraction. These increases were mostly at the expense of free aneurin which was decreased by thyroxine injection. Thiouracil changed the partition of aneurin in the opposite way. There were close negative correlations between phosphatase and cocarboxylase. Likewise there were close negative correlations between phosphatase and protein-bound aneurin. The correlations between phosphatase and free aneurin were positive. These correlations were found in all cows, irrespective of treatment with thyroxine or thiouracil. Implications of these correlations in the biochemistry of milk secretion have been discussed.

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Uptake of Potassium Tellurite by a Sensitive Strain of *Escherichia coli*

By P. D. COOPER AND A. V. FEW

Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, Paddington, W. 2

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As early as 1913 Schurmann & Hajos added potassium tellurite to a differential medium for the isolation of the diphtheria bacillus, and this use of potassium tellurite has now become familiar. Cavazutti (1921) and Joachimoglu (1920, 1922) found that in general Gram-positive organisms were less affected by tellurite than Gram-negative, and that the activity of related compounds decreased in the order $\text{TeO}_3^{2-} > \text{SeO}_3^{2-} > \text{TeO}_4^{2-} > \text{SeO}_4^{2-}$. Fleming (1932, 1942) demonstrated the value of potassium tellurite when used with penicillin and gentian violet for the isolation of certain bacteria from badly contaminated sources. Penicillin and potassium tellurite were both active at high dilution on entirely different bacterial species so that, with a few exceptions, bacteria which were penicillin-sensitive were tellurite-resistant and vice versa. Strains of *Escherichia coli* sensitive to tellurite could rapidly acquire resistance by serial subcultivation in increasing concentrations of the salt (Fleming & Young, 1940) and the antigenic and fermentation characteristics of both sensitive and naturally resistant strains of *Esch. coli* appeared to be identical. There was also some evidence of a very high absorption of tellurite by actively growing bacteria. Growth on tellurite agar yields black colonies, and granules can be seen inside the cells which are presumably composed of elementary tellurium (Hewitt, 1951).

In the present communication, as an approach to the means by which potassium tellurite (K_2TeO_3) inhibits bacterial growth, the mechanism of assimilation of potassium tellurite by bacterial suspensions is studied by the quantitative determination of the uptake using ^{127}Te incorporated into potassium tellurite as radioactive tracer.

Although the tellurium was added to the bacterial suspensions as the alkaline salt K_2TeO_3 , the true substrate of the assimilation process at pH 5.5 may be TeO_2 , H_2TeO_3 or HTeO_3^- . The pK_a values of the two dissociation stages of tellurous acid were found to be 8.9 and > 10 , so that only about 0.1% of H_2TeO_3 is ionized at pH 5.5. Whatever the nature of this substrate, however, or of the final chemical form in which Te accumulates in the cells, uptakes and concentrations have been expressed throughout in terms of K_2TeO_3 .

METHODS

Preparation of radioactive potassium tellurite

Acidic residues, containing radioactive Te obtained in the preparation of $^{131}\text{I}_2$, were supplied by the Atomic Energy Research Establishment, Harwell, with an activity of approximately 5 mc. in 100 ml. Oxalate, sulphate and chromic ions were also present, and elementary Te was slowly precipitated from the acid solution by the addition of 10 g. of $\text{Na}_2\text{S}_2\text{O}_5$ to 50 ml. of residues. After 48 hr. the precipitation appeared to be complete and the Te was separated and washed five times with distilled water. The moist Te was dissolved in a slight excess of 50% (v/v) HNO_3 , and the solution was cleared by centrifuging and diluted with distilled water to 6 ml. On addition of 20 ml. of ethanol a flocculent white precipitate of TeO_2 appeared. After standing overnight this was washed three times with distilled water in the centrifuge and dried for 24 hr. *in vacuo* over P_2O_5 . The product was weighed, and the theoretical amount of 1% (w/v) KOH solution added to form K_2TeO_3 . The solution was finally diluted to a concentration of 1000 $\mu\text{g. K}_2\text{TeO}_3/\text{ml.}$

The overall yields of K_2TeO_3 were 100–150 mg., with an initial specific activity of about 0.5 $\mu\text{c./mg.}$ and a radioactive recovery of 2%. The half life of this solution corresponded exactly with that of ^{127}Te (90 days).

The concentration of the radioactive K_2TeO_3 solution was then confirmed by the colorimetric method of Shakhov (1945) and by isotope dilution with a standard 0.1% (w/v) K_2TeO_3 solution.

These determinations showed that the saturation concentration of K_2TeO_3 at 18° and pH 5.5 was 120–150 μ g./ml. Accordingly, no experiments were conducted at a higher concentration than 100 μ g./ml.

As a sample of commercial potassium tellurite was found to contain more than 30% excess of the theoretical quantity of TeO_2 , the standard solution of K_2TeO_3 used above was prepared from dried TeO_2 obtained from the commercial salt by precipitation with acid.

Preparation of bacterial suspensions

One of the organisms most sensitive to tellurite among ten strains tested was found to be *Esch. coli* D. 433, from the National Collection of Type Cultures, and this has been used throughout these experiments. Saline suspensions were prepared from a 16 hr. agar slope, and used for inoculation of 250 ml. of the semi-synthetic medium described below. After incubation in Roux bottles at 37° for 16–18 hr., the cultures were harvested and washed three times with distilled water at 4°, yielding approx. 60 mg. dry wt. of bacteria/250 ml. medium.

1 l. of the medium contained 1 g. KH_2PO_4 , 0.7 g. $MgSO_4 \cdot 7H_2O$, 1 g. NaCl, 4 g. $(NH_4)_2HPO_4$, 0.5 g. trisodium citrate, and 0.5 mg. $FeCl_3$, together with 100 ml. of a neutralized acid casein hydrolysate containing 14.2 mg. N/ml. and 9.1 mg. PO_4^{3-} /ml., prepared by the method of Mueller (1939). NaOH solution was added to pH 6 and the medium was autoclaved in 250 ml. screw-capped bottles. The pH was unchanged by sterilization and growth.

Measurement of uptake

The procedure was similar to that previously used in this laboratory for the determination of the uptake of radioactive penicillin by bacteria (see Rowley, Cooper, Roberts & Smith, 1950). The washed bacterial suspensions, at approximately the required concentration, were aerated for 1 hr., as this procedure was found to increase tellurite uptake. A 0.1 ml. sample was diluted to 10 ml. for assay of bacterial dry wt./ml. on a calibrated Hilger absorptiometer. Samples (1 ml.) in centrifuge tubes were then incubated with buffer solution for 15 min. before addition of K_2TeO_3 and further buffer up to 5 ml. After a given time at 37° the tubes were cooled in ice-water, centrifuged rapidly (8 min. at 3500 g) and washed three times in distilled water after which the final supernatant solutions were entirely free of radioactivity. Care was taken not to remove bacteria with the supernatant solution. The washed bacteria were transferred to calibrated tubes and made up to 2 ml. with distilled water, and two 0.25 ml. samples were allowed to dry on planchettes for radioactive assay. As no attached radioactivity was found to be lost by the washing process, the uptake could then be calculated in terms of μ g. of K_2TeO_3 /mg. bacterial dry wt., knowing the specific activity of the K_2TeO_3 and the initial bacterial concentration.

Phosphate buffer (0.03M) at the optimum pH (5.5) and temperature (37°) was employed, unless otherwise stated. Preliminary experiments were made to ensure that the total amount of tellurite removed from the solution did not decrease its concentration by more than 3%, which was regarded as an insignificant decrease as its effect was well

within experimental error. This generally involved the use of bacterial concentrations <2 mg./ml. with tellurite concentrations of 40 μ g./ml. and a reaction time of 45 min.

Radioactive assay

This was performed on the same apparatus used previously (Rowley *et al.* 1950). Total counting rates were generally of the order of 100/min., and duplicate planchettes were each counted for two 10 min. periods at different times during the day. The probable counting error is approx. $\pm 2.0\%$ for each sample. A background count (approx. 14/min.) was taken for 60–80 min. at midday and the value obtained was subtracted from the average total count for each sample. A uranium standard planchette was counted daily at three different voltages to determine plateau stability, together with a Te standard three times during the day. With Te no self-absorption correction was found to be necessary with the layer thicknesses used. Allowance was made for radioactive decay when calculating the weight of K_2TeO_3 bound by the cells.

The total error associated with the bacterial uptakes of radioactive Te was found to be approximately $\pm 10\%$, composed of the counting error ($\pm 2\%$), the absorptiometer error of $\pm 3\%$ and the sampling error.

RESULTS

Non-removal of ^{125}Te from Escherichia coli by washing with distilled water. The amount of ^{125}Te attached to the organisms after incubation with radioactive K_2TeO_3 , but before the organisms were washed, was calculated from the difference between radioactive assays of the bacterial suspension of known bacterial concentration and the supernatant solution after centrifuging. The supernatant solution was discarded and the bacteria resuspended to the same concentration as initially. The suspension was then re-assayed, centrifuged, and the supernatant re-assayed. This was repeated several times and the uptakes were found to be constant within experimental error.

The effect of temperature variation. The uptake of K_2TeO_3 by *Esch. coli* was found to be dependent upon the temperature at which the reaction between the bacterial suspensions and K_2TeO_3 occurred. Fig. 1 shows that the uptake was maximal at about 37°, falling off at higher or lower temperatures.

The activity of the bacteria in suspensions at different pH, and in different buffers. Fig. 2 shows the effect of variation of pH in the suspending medium on the uptake of K_2TeO_3 .

The activity was maximal at pH 5.5 whether the sample was aerated beforehand or not, although aeration markedly increased the uptake by the organisms. A mixture of 0.03M-acetate and 0.03M-phosphate buffers was employed to ensure pH stability over the range 4.7–6.5. The use of 0.03M-acetate, phosphate and succinate buffers gave approximately the same uptake at the optimum pH, but citrate buffer lowered the uptake by about

75 %. Sodium citrate was also effective in preventing the precipitation of TeO_2 from K_2TeO_3 between pH 3 and 7 and it seems likely that a complex is formed between these salts.

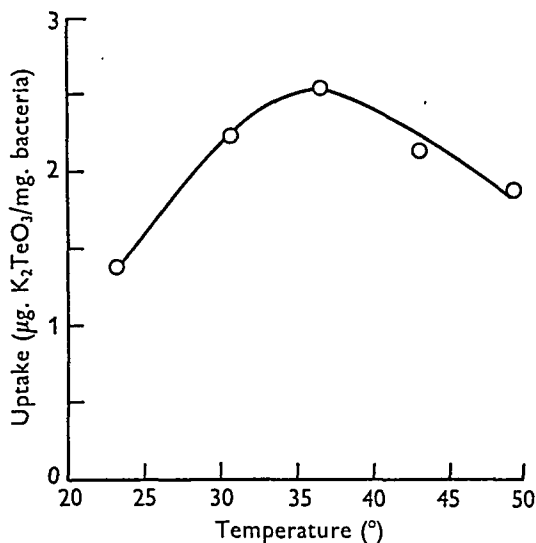


Fig. 1. Uptake of K_2TeO_3 on *Esch. coli* after incubation for 45 min. at different temperatures in 0.03M-phosphate (pH 5.5) containing 40 µg./ml. K_2TeO_3 .

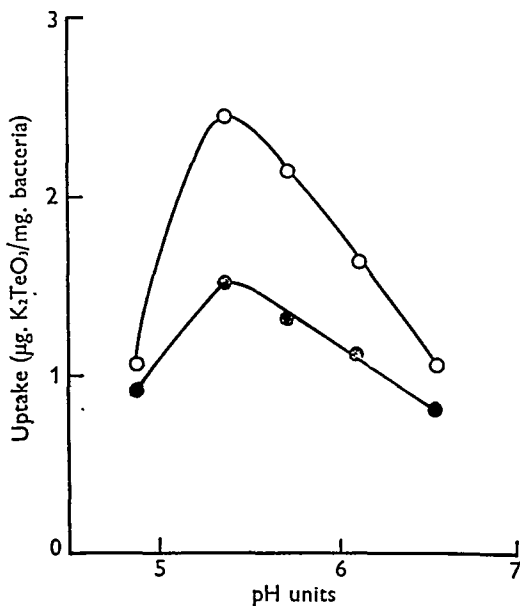


Fig. 2. Variation of uptake of K_2TeO_3 on *Esch. coli* with pH of the suspending medium. Organisms were incubated for 45 min. at 37° in a mixture of 0.03M-phosphate and 0.03M-acetate containing 40 µg./ml. K_2TeO_3 . O—O, one sample of bacteria aerated for 1 hr. at 18° before incubation; ●—●, the residue allowed to stand in contact with air at 18° concurrently.

In general, therefore, when experiments were performed at pH 5.5, 0.03M-phosphate buffer was employed as this seemed without effect on the uptake.

Other factors affecting the activity of the system responsible for tellurite assimilation. The exposure of the bacterial suspensions to low pH for 1 hr. at 37° before adjusting to pH 5.5 caused a considerable decrease in the power of the organisms to assimilate K_2TeO_3 (Fig. 3). No loss of activity occurred at

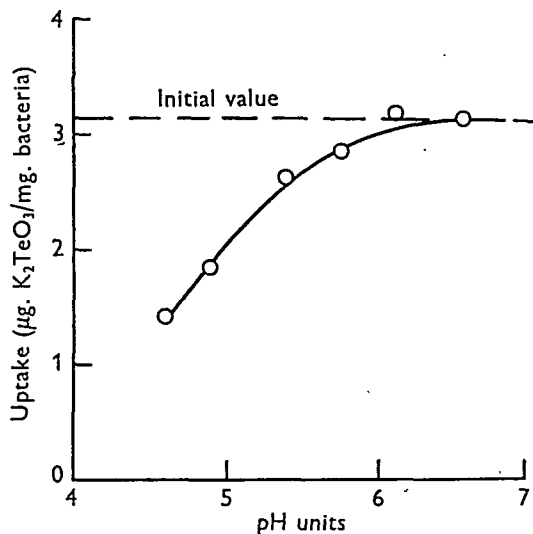


Fig. 3. Effect of pH on the stability of the system in *Esch. coli* responsible for tellurite uptake. Organisms were suspended in a mixture of 0.03M-phosphate and 0.03M-acetate for 1 hr. at 37°, centrifuged rapidly and re-suspended in warm 0.03M-phosphate at pH 5.5. K_2TeO_3 was then added to 40 µg./ml. and the uptake was assayed after a further 45 min. at 37°.

pH 6-7 and only about 10 % at the working pH of 5.5. When the activity of the organisms was determined after different times of incubation at pH 5.5 there appeared to be a slight loss after several hours, but during the uptake period of 45 min. usually employed the loss was negligible (Table 1).

Table 1. Rate of inactivation at pH 5.5 of the system responsible for the uptake of K_2TeO_3 by *Esch. coli*

(K_2TeO_3 was added to a concentration of 40 µg./ml. after varying times of incubation at pH 5.5, and the radioactivity on the bacteria was assayed after a further 45 min. at 37°.)

Time (hr.)	Uptake (µg./mg.)
0	3.3
1	3.3
2.25	3.1
3.5	2.9
4.75	2.3
22	1.5

As bacteria contain easily oxidizable substances which interfere with processes such as methylene-blue reduction (Quastel & Whetham, 1925) it seemed advisable to determine the effect of removal of these substances before the addition of K_2TeO_3 ,

since this substance is also easily reduced. Accordingly, air was rapidly bubbled through the bacterial suspensions for 1 hr. at room temperature before commencing the experiment. In contrast with the effect on the rate at which methylene blue was reduced by bacteria, aeration was found to increase the tellurite uptake whilst treatment with nitrogen decreased it in relation to a suspension left in contact with air (Table 2). Treatment with nitrogen for 1 hr. after aeration gave uptake values which were identical with those of the aerated organisms.

Table 2. *Effect of aeration on the uptake of K_2TeO_3*

(Portions of an *Esch. coli* suspension at pH 5.5 were simultaneously stood in air, bubbled with air and bubbled with N_2 respectively for 1 hr. at room temperature. Half of the aerated sample was then bubbled with N_2 for a further hour, K_2TeO_3 was added to 40 $\mu g./ml.$ to all four samples and the uptake was measured after a further 45 min. at 37° during which time the N_2 treatment was continued.)

Treatment	Uptake ($\mu g./mg.$)
N_2 bubbled	1.6
Stood in air	2.3
Air bubbled	2.8
Air bubbled for 1 hr. then N_2 for 1 hr.	2.7

The effect of the inclusion of glucose in the growth medium on the ability of *Esch. coli* to assimilate tellurite. Glucose was at first included in the growth medium in order to increase the yield of *Esch. coli*, but it was found that the resultant organisms had a lowered capacity to take up K_2TeO_3 . Fig. 4 shows that as little as 0.05% glucose markedly inhibited the formation of the tellurite-absorbing system so that the uptake was lowered whether the organisms were aerated before incubation with K_2TeO_3 or not. At this low glucose concentration the pH of the growth medium was scarcely altered after growth of the organisms so that the inhibition was not due to decomposition caused by fermentation acids before harvesting.

The further decrease in activity at higher glucose concentrations may be due to decomposition caused by acidity.

The effect of variation of the bacterial concentration on the uptake. When K_2TeO_3 at 40 $\mu g./ml.$ was incubated for 45 min. at 37° with varying concentrations of *Esch. coli*, the uptake per mg. dry weight was found to be independent of the bacterial concentration, when this was less than 2 mg./ml. The uptake decreased at higher values, no doubt owing to the removal of a significant fraction of the K_2TeO_3 during uptake.

Variation of uptake with time of contact with tellurite. The amount of K_2TeO_3 attached to *Esch. coli* increased rapidly with time of incubation at 37°, reaching a maximum at 3–4 hr. and decreasing to

a very low value after 22 hr. (Fig. 5). The rate of uptake was much less at 1°.

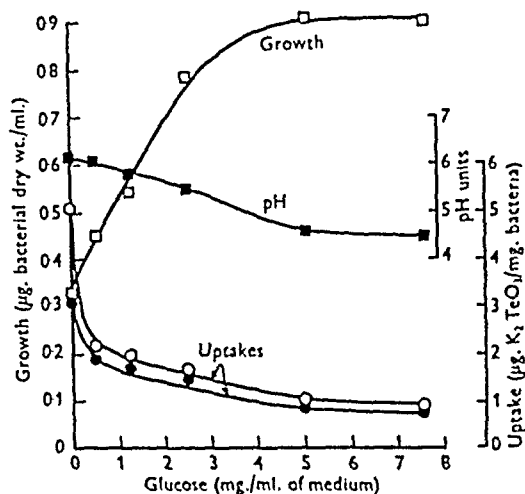


Fig. 4. Effect of inclusion of glucose in the growth medium on the ability of *Esch. coli* to take up K_2TeO_3 , after being washed free from the medium. Organisms were incubated at 37° for 45 min. in 0.03M-phosphate (pH 5.5) containing 40 $\mu g./ml.$ K_2TeO_3 . □—□, bacterial growth in the medium after 16 hr. incubation; ■—■, pH after growth (initial value 6.0); ○—○, uptake after prior aeration for 1 hr. at 18°; ●—●, uptake after standing for 1 hr. at 18° without aeration.

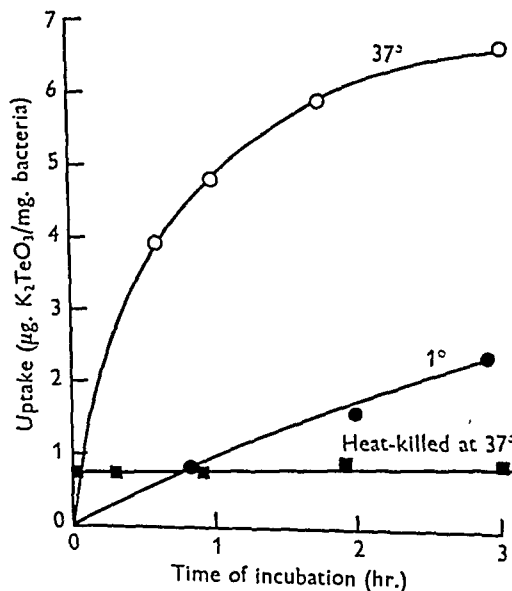


Fig. 5. Rate of uptake of K_2TeO_3 on aerated samples of *Esch. coli* in 0.03M-phosphate containing 40 $\mu g./ml.$ K_2TeO_3 . ○—○, live organisms at 37°; ●—●, live organisms at 1°; ■—■, heat-killed organisms at 37°.

Variation of rate of uptake with concentration of tellurite. The amount of K_2TeO_3 attached to *Esch. coli* increased with the concentration of K_2TeO_3 in

solution in a manner shown in Fig. 6. When the reciprocal of the rate of uptake at 37° was plotted against the reciprocal of the tellurite concentration, following the procedure of Lineweaver & Burk (1934) for enzyme systems, a straight line resulted. Thus the relationship between the rate of uptake and the tellurite concentration was the same as that between the rate of an enzyme-catalysed reaction and its substrate concentration. The Michaelis constant (K_m) was calculated from this straight line by the method of Lineweaver & Burk.

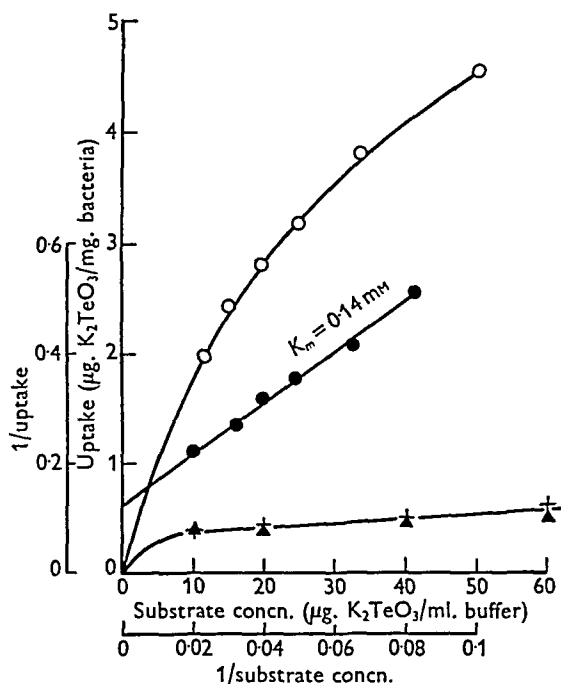


Fig. 6. Variation of uptake of K_2TeO_3 on *Esch. coli* with tellurite concentration. Organisms were incubated at 37° for 45 min. in 0.03M-phosphate buffer (pH 5.5) containing K_2TeO_3 . ▲—▲, heat-killed organisms un-aerated; +—+, heat-killed organisms aerated for 1 hr.; ○—○, live organisms aerated for 1 hr.; ●—●, plot of reciprocal uptake against reciprocal tellurite concn. for the experiment using live organisms.

Table 3 shows that such experimental values of the Michaelis constant obtained varied between rather wide limits, but within these limits no difference was noticed whether the organisms were previously (a) grown in glucose, (b) un-aerated, (c) aerated or (d) aerated and subsequently bubbled with nitrogen. Procedures (a) and (b) significantly decreased the activity of the organisms compared with the procedures (c) and (d).

The solubility of K_2TeO_3 at pH 5.5 was too low to allow the K_m to be derived by the alternative method of saturation of enzyme with substrate.

Effect of heat treatment. Immersion of a suspension of *Esch. coli* in a boiling-water bath for 20 min.,

followed by rapid cooling, altered the rate of uptake by the organisms (Fig. 5). A small amount of tellurite was taken up very rapidly, but after this no further uptake occurred. The amount of tellurite absorbed was also much less dependent upon concentration of the salt (Fig. 6), and the site of attachment appeared nearly saturated at quite low tellurite concentrations. Prior aeration had no effect.

Table 3. Values for the Michaelis constant of the system responsible for K_2TeO_3 uptake on *Esch. coli*

K_m (mM)			
Aerated organisms	Un-aerated organisms	Aerated and bubbled with N_2	0.05% Glucose added to the growth medium, aerated
0.25	0.23	0.25	0.39
0.094	0.67	—	0.16
0.14	—	—	—
0.14	—	—	—
0.47	—	—	—

DISCUSSION

It has been shown above that potassium tellurite is rapidly assimilated by *Esch. coli* in amounts up to 1% of its dry weight and calculation shows that the concentration in the bacteria can reach 20 times the concentration in the medium. There is thus some mechanism allowing active transfer from the medium into the cells, and it appears very likely that this mechanism is associated with an enzyme or a group of enzymes. However, as Slater (1949) has pointed out, the fact that application of the Lineweaver & Burk equation gives a straight line is not conclusive evidence of an enzymic reaction, nor does this fact show whether or not an enzymic uptake has superimposed upon it an uptake of constant amount similar, for example, to that fixed by heat-killed organisms.

If the organisms were first killed by heat the uptake was radically altered, being much lower and much less dependent on K_2TeO_3 concentration, pH, time and temperature. The uptake curves in this case suggest a rapid chemical combination with the bacteria which is completed at relatively low tellurite concentrations.

The lowered activity when the organisms are grown with glucose suggests that the formation of the enzyme is inhibited in a similar manner to that reported by Epps & Gale (1942) for the formation of several enzymes in *Esch. coli* including certain deaminases and dehydrogenases. In these cases, as with the tellurite absorbing system, only a small part of the inhibition was due to the low pH in the medium induced by glucose.

As passage of nitrogen through the suspensions does not increase activity, the increase caused by aeration is unlikely to be due to the removal of volatile inhibitors. The activation is thus presumably due to the chemical action of oxygen and is not neutralized by the subsequent passage of nitrogen.

It is interesting that this aeration of the suspension before addition of K_2TeO_3 (a process employed by Quastel & Whetham (1925) to remove reducing substances before addition of methylene blue in Thunberg experiments) actually increased the rate of uptake of tellurite. The uptake of K_2TeO_3 , which appears to involve a reduction to tellurium, may be similar to the reduction of methylene blue by bacteria observed in the absence of substrate in the Thunberg estimation. Both reactions use up hydrogen donors which are still present in the bacteria after washing and aeration, and one cause of the stimulation of tellurite uptake by oxygen may be due to the inhibition by this gas of alternative processes which utilize and remove the intracellular 'H' donors. This is similar to a suggestion made by Stephenson (1949) as an explanation of the Pasteur effect where yeast fermentation is repressed by oxygen in favour of complete oxidation of the substrates to carbon dioxide.

SUMMARY

1. By incorporating ^{127}Te into potassium tellurite (K_2TeO_3), the amount of the salt absorbed by a buffered suspension of a tellurite-sensitive strain of *Escherichia coli* could be calculated from a measure of the radioactivity remaining on the washed organisms. No radioactivity was lost by washing.

2. The system responsible for tellurite uptake was unstable at low pH but did not deteriorate greatly over several hours at pH 5.5-7.

3. The uptake of tellurite by the organisms increased with time, and was most rapid at pH 5.5 and 37°. The uptake had the properties of an enzyme action.

4. Growth of *Esch. coli* in a medium containing 0.05% glucose decreased the activity of the organisms, while the pH of the medium was scarcely affected.

5. Heating to 100° for 20 min. destroyed the enzymic system. The low uptake then obtained corresponded to a rapid combination with the cells which were saturated at fairly low tellurite concentrations.

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Antibiotic Peptides from *Bacillus licheniformis*. Licheniformins A, B and C

By R. K. CALLOW AND T. S. WORK

The National Institute for Medical Research, Mill Hill, London, N.W. 7

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From the spore-forming aerobe *Bacillus licheniformis* Weigmann *emend.* Gibson, grown on an ammonium and sodium lactate medium, an antibiotic material, licheniformin, was prepared (Callow, Glover, Hart & Hills, 1947). It was obtained as the hydrochloride of basic material containing only carbon, hydrogen and nitrogen. It had bacteriostatic activity *in vitro* against numerous organisms, notably *Mycobacterium tuberculosis*, and protective effects against infections, including tuberculous infection, in mice. It was, however, toxic, causing damage to the kidneys after prolonged administration.

Further purification and chemical investigation was of importance from the practical point of view, to see whether the toxicity was inherent or could be separated from the antibiotic activity by fractionation. From the theoretical point of view, the constitution of a substance able to suppress experimental tuberculous infection was of the greatest interest. The crude active material for long resisted attempts at fractionation, but this was at last accomplished after it was found that separation into components could be effected by paper chromatography. This separation was used as a guide in devising a suitable scheme of counter-current fractionation on a preparative scale.

The separation of three components, designated licheniformins A, B and C, is now described. They have proved to be peptides of very similar molecular weights and amino-acid composition, all having both antibacterial activity and toxicity, though to somewhat varying degrees.

Taken in conjunction with results obtained at the same time at two other centres of research, the Microbiological Research Department, Porton, and the Sir William Dunn Institute of Pathology, University of Oxford, the present results show that a single species of bacterium can produce at least six antibiotic polypeptides, one set of three, the licheniformins, on a medium with lactate and a high nitrogen/carbon ratio, and another set of three, the bacitracins, on a medium with glucose and a low nitrogen/carbon ratio. The late G. M. Hills collaborated not only with ourselves but also with the Oxford laboratories in devising suitable media for bulk production of antibiotic peptides from *B.*

licheniformis and recognized the decisive influence of the medium on the type of antibiotic produced (Callow *et al.* 1947; Hart & Hills, 1947; Belton, Hills & Powell, 1949; Hills, Belton & Blatchley, 1949; Arriagada, Savage, Abraham, Heatley & Sharp, 1949).

EXPERIMENTAL

MATERIALS AND GENERAL METHODS

Organisms and culture conditions

We are indebted to the Microbiological Research Department, Porton, for culture fluids from the original strains of *B. licheniformis*, National Collection of Type Cultures (N.C.T.C.) no. 7072, and from the Oxford 'A5', and to Boots Pure Drug Co. Ltd. for culture fluids from N.C.T.C. nos. 5399 and 6816, and from 'Plate 2C', another strain isolated by Dr P. D'Arcy Hart. Culture was on the medium previously described, or modifications of this (Callow *et al.* 1947; Hart & Hills, 1947; Belton *et al.* 1949).

Extraction of licheniformin

The processing of early batches was carried out as described previously (Callow *et al.* 1947). Later, however, after experiments with different adsorbents, it was found that Decalso F (Permutit Co. Ltd., London), a sodium aluminosilicate, acted as an adsorbent of high capacity from which licheniformin was readily eluted by NaCl solution. The procedure described below is more rapid and convenient than the active-charcoal process.

The supernatant from the autoclaved and centrifuged culture was brought to pH 5-6 and further clarified, if necessary, by filtration through a layer of Hyflo Supercel (Johns-Manville Co. Ltd., London) and then run on to a dry column containing 5 g. of Decalso F for each litre of liquid. A convenient column was provided by an inverted Winchester-quart bottle with the bottom removed and a bung and tap fitted in the neck. Fluid was fed from a large aspirator bottle, closed at the top and with a wide-bore tap and bent delivery tube from the lower tubulure dipping into the liquid above the column, so forming a constant-level device. The rate of flow was 0.3-1.0 l./hr. When the fluid level finally fell to the level of the top of the adsorbent 10% (w/v) NaCl solution was run on as eluent. The rate of flow was decreased to about 2 drops/sec. and the effluent was tested at intervals by adding 1 drop to 0.5 ml. of saturated aqueous solution of picric acid. When a definite precipitate was formed, collection of the eluate was begun and continued until the picric acid test became very weak. About 50 ml. of 10% NaCl was sufficient to elute the licheniformin

present in 1 l. of culture fluid of normal potency (500–1000 units/ml.). A slight excess of picric acid solution was added to the eluate, the precipitate was allowed to settle and collected on a sintered-glass filter (porosity 3). The picrate was decomposed on the filter by grinding with conc. HCl, the solution sucked through and run into a large excess of dry acetone with stirring. Crude licheniformin hydrochloride was precipitated. It was collected and dried *in vacuo* and further purified by solution in methanol (4 ml./g.), filtration, and reprecipitation by pouring into 25 vol. of acetone. The usual yield was 40–50 mg. of material with an activity of between 5 and 10×10^6 units/g. from 1 l. of culture fluid. With the rather inaccurate bio-assay method used, no significant loss of activity was detected during isolation.

Paper chromatography

Amino-acid separations were carried out as described by Dent (1948). The special solvent mixture for separation of licheniformin is described below.

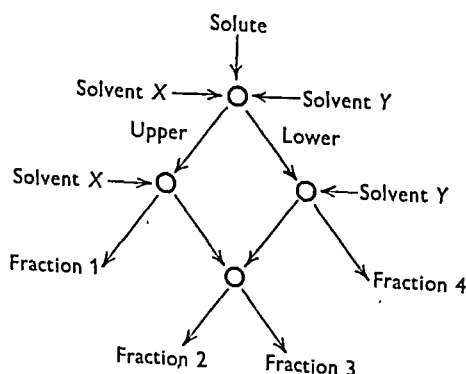
Antibacterial activity

This was estimated by the serial dilution method previously described (Callow *et al.* 1947) using *Mycobacterium phlei* as the test organism. We are indebted to Dr P. D'Arcy Hart for the numerous assays made in the course of this work.

SEPARATION OF LICHENIFORMINS

Counter-current distribution between immiscible solvents

The system of fractionation described by Bush & Densen (1948) was used. In this n funnels, with final complete separation of the phases, yield $2n$ fractions. The system may be simply explained by means of a diagram showing the use of two funnels:



Bush & Densen (1948) give an equation which can be used to calculate the composition of the fractions in terms of the number of funnels used and the proportions of a substance in the upper and lower layers, p and q , respectively, where $p + q = 1$. For a 12-funnel separation giving 24 fractions, these have the compositions: p^{12} , $12p^{11}q$, $78p^{10}q^2$, $364p^9q^3$, $1365p^8q^4$, $4368p^7q^5$, $12376p^6q^6$, $31824p^5q^7$, $75582p^4q^8$, $167960p^3q^9$, $352716p^2q^{10}$, $705432p^1q^{11}$, $705432p^0q^{12}$, $352716p^1q^{11}$, $167960p^2q^{10}$, $75582p^3q^9$, $31824p^4q^8$, $12376p^5q^7$, $4368p^6q^6$, $1365p^7q^5$, $364p^8q^4$, $78p^9q^3$, $12p^{10}q^2$, q^{12} . Using

selected values of p and q a family of curves was drawn. By comparison of experimental graphs of weights of fractions against number in the series 1–24 it was then possible to estimate roughly the relative amounts and partition coefficients of major components of mixtures, by comparison of the heights and positions of the experimental maxima with the theoretical curves. The goodness of fit of a theoretical curve to the experimental curve for what appeared to be a single substance enabled one to judge its homogeneity with respect to partition coefficient. Bush & Densen also give an expression for the optimal proportions of upper and lower layers for separation of substances of known different partition coefficients. Thus, application of the procedure to separations on a preparative scale was simple over a range of partition coefficients, with appropriate alteration of the relative volumes of the two layers. At first ordinary separating funnels were used; later an automatic all-glass separating apparatus, as described by Craig & Post (1949), was constructed. This had 12 tubes each with a capacity of 92 ml. for the lower layer, and a maximum capacity of about the same for the top layer.

Paper chromatography

A large number of solvent mixtures were tried on one-dimensional paper partition chromatograms with descending flow. The only one effective in fractionating crude licheniformin was a mixture of collidine, lutidine and aqueous 2N-ammonia (1:1:2 by vol.). After vigorous shaking and separation, the upper phase was used in the trough and the lower used to saturate the atmosphere of the tank in which the chromatograms were run. Whatman no. 4 paper was used throughout. Spots were detected with ninhydrin spray in the way usual for amino-acids.

The procedure described below demonstrated the presence of three chromatographically distinct antibiotic components.

Licheniformin hydrochloride (1 mg.) was dissolved in water and transferred to four equally spaced spots at the top of a sheet of paper (length 50 cm.). The paper was irrigated with the upper layer of the solvent mixture. The solvent reached the end of the paper in 24 hr. and was allowed to run off the end of the sheet for another 24 hr. The paper was dried, washed thoroughly with ether until free of the odour of collidine and cut into four strips, each with one of the original spots at the top. Two of the strips were treated with ninhydrin and each was found to show several distinct zones of colour (Fig. 1). The four strips were laid side by side and the probable positions of the ninhydrin-positive spots marked in pencil on the untreated strips. The two untreated strips were now laid on the surface of two large agar plates (30 × 20 cm.) which had been previously poured with a uniformly distributed inoculum of licheniformin-sensitive bacteria, *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) or *Mycobact. phlei* (cf. Goodall & Levi, 1946). The plates were left at 0° for about 12 hr. and then incubated at 37° for 6 hr. (*Staph. aureus*) or 24 hr. (*Mycobact. phlei*). At the end of this time the paper was stripped from the surface of the agar and the plate examined for zones of inhibition (clear zones). The type of result which was obtained is shown in Fig. 1. The three clear zones on each agar plate corresponded with three coloured zones on the papers sprayed with ninhydrin. The three antibiotics separated by this procedure were named licheniformins A, B and C in order of increasing R_F values.

Unsuccessful attempts at fractionation

With paper-chromatographic analysis as a guide, various methods of larger-scale fractionation were tried. Fractional precipitation from aqueous solution by $(\text{NH}_4)_2\text{SO}_4$ or by methyl orange did not result in any separation. Activity was adsorbed by acid-washed alumina from methanolic solution, and material could then be eluted by aqueous methanol of increasing water content, but paper chromato-

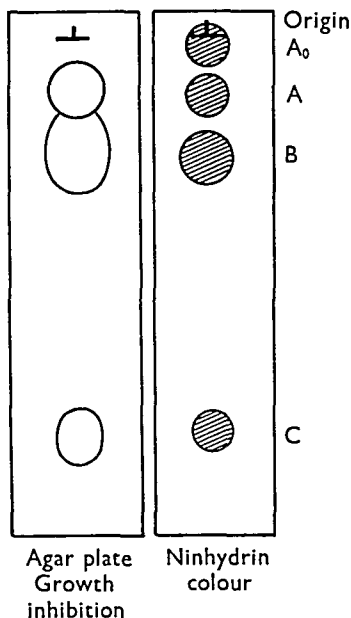


Fig. 1. Separation of three antibiotics by paper chromatography of crude licheniformin (for method see text). Zones of inhibition of bacterial growth are indicated by unshaded ovals. Zones coloured after spraying with ninhydrin are hatched.

graphy of the eluates showed that separation was far from complete. Elution of licheniformin adsorbed on Decalso F by dilute NaCl also gave only partial separation. Chromatography on a starch column in a mixture of collidine, lutidine and ammonia gave no evidence of fractionation. Ionophoresis in silica jelly (Consden, Gordon & Martin, 1946) promised interesting results, for under certain conditions three opalescent bands moved towards the cathode. It was, however, impossible completely to separate these bands, which tailed and became diffuse as they travelled, and little antibiotic activity could be recovered.

Application of counter-current distribution

Several solvent systems were tried for the fractionation method outlined above. Licheniformin, as the hydrochloride, is stable but extremely soluble in water, and insoluble in the common organic solvents. Alkali metal hydroxides decompose licheniformin, but decomposition was not rapid in Sorensen borate-NaOH buffer at pH 12.38, and *n*-butanol extracted some activity from such a solution. *n*-Butanol and 8.5*N*- NH_3 solution, *sec.*-butanol and 4.25*N*- NH_3 solution and *n*-butanol, water, and piperidine (2:2:1 by vol.) all gave two liquid phases with licheniformin in each and were potentially useful for fractionation by partition,

but they were unpleasant. Ultimately it was found that, in a mixture of phenol and water, a specimen of crude licheniformin had a partition coefficient (*K*) of 2.27 (ratio of concn. in upper water layer to concn. in lower layer); emulsification was largely prevented by addition of a little HCl. This system was used successfully.

The phenol-water mixture was prepared by melting the phenol ('detached crystals') and adding the appropriate amount of warm water. HCl (5 drops, about 0.25 ml., of 12*N*/l.) was added, and the mixture equilibrated at room temperature. The crude licheniformin hydrochloride was dissolved in upper layer and the solution made

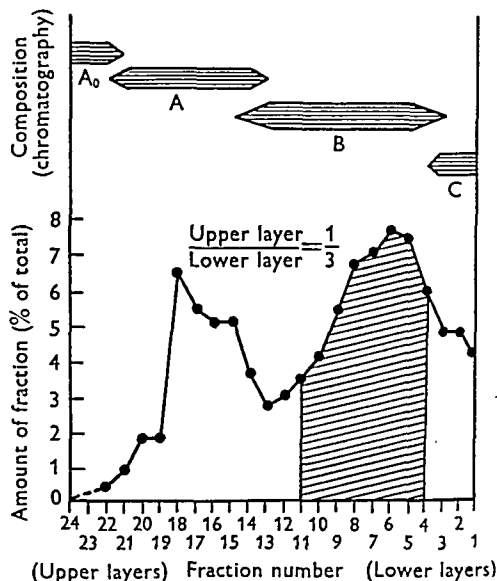


Fig. 2. Counter-current fractionation diagram for crude licheniformin hydrochloride partitioned between water (containing HCl) and phenol (for quantities see text). \square indicates material selected for refractionation to separate licheniformin B. The contents of each tube were also analysed by paper chromatography and the qualitative composition of the product is indicated diagrammatically above the curve.

up to the required volume and then added to the funnel containing lower layer; this constituted the 'first funnel' of the counter-current extraction. The final fractions were 12 water-rich upper layers and 12 phenol-rich lower layers. Recovery of material from these was at first done by way of the picrates, but this step was unnecessary and the following procedure was adopted, e.g. with 25 ml. upper layer and 75 ml. lower layer. Phenol was extracted from the upper layer with 80 ml. ether which was back-extracted with 2×20 ml. water. The combined water extracts were evaporated to dryness under reduced pressure; the residue was taken up in a little methanol and the solution poured into excess of acetone. The precipitated material was collected by centrifugation in a weighed 15 ml. tube, dried, and weighed. The lower layer was diluted with 20 ml. water and extracted with 200 ml. ether, which was back-extracted with 2×20 ml. water. The combined water extracts were then evaporated and material precipitated as before. Recovery was usually about 90% of the initial weight. Direct precipitation by pouring the layers into acetone was possible, but used much acetone.

The weighed fractions were examined by paper chromatography and a combined diagram could then be constructed in which weights and paper-chromatographic analyses were plotted against fraction numbers, as in Fig. 2, which gives results obtained with 2.14 g. of crude licheniformin hydrochloride prepared by the Decalso process from N.C.T.C. no. 5399 and partitioned between 12.5 ml. upper and 37.5 ml. lower layers (abbreviated as water/PhOH = 1/3).

The next step in the isolation of one of the constituents was to select fractions which were chromatographically homogeneous, or nearly so, with respect to the desired constituent. These were united and again submitted to counter-current fractionation. In doing this the ratio of

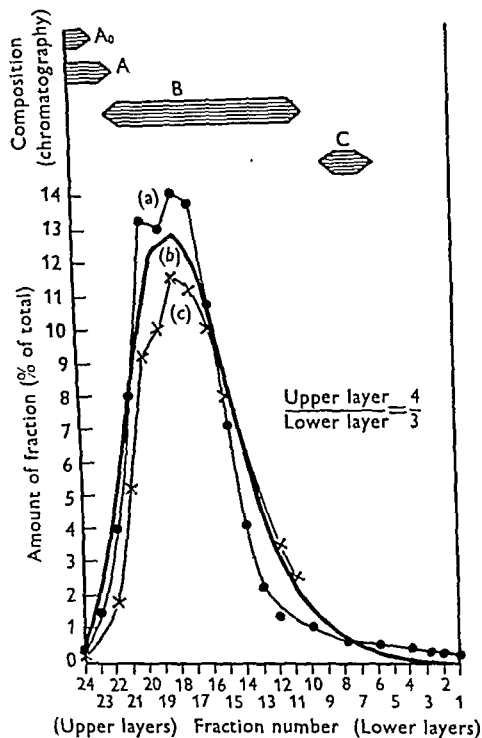


Fig. 3. (a) Counter-current fractionation diagram for partially purified licheniformin B hydrochloride partitioned between water (containing HCl) and phenol. The theoretical curve for $K=1.33$, $p=0.64$ with upper layer/lower layer = 4/3 is shown as a thick line (b). The qualitative compositions (paper chromatography) of the fractions are indicated diagrammatically above the curves. Curve c was obtained after two further counter-current fractionations of material represented by curve a (for details see text).

upper and lower layers was altered so that the separation of the desired constituent from an adjacent one was more complete, e.g. alteration of the ratio water/PhOH to 1/0.75 brought constituent B into the upper layers with a maximum weight in fraction 18 and C was largely confined to the phenol layers. This is illustrated by curve a in Fig. 3, giving the results of taking fractions 4–11, wt. 0.92 g., from the previously illustrated fractionation (Fig. 2) and partitioning between 40 ml. upper and 30 ml. lower layer. A theoretical

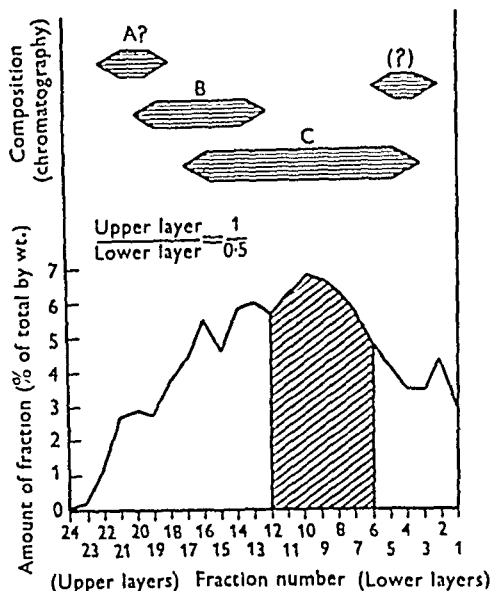


Fig. 4. Counter-current fractionation diagram for crude licheniformin C hydrochloride partitioned between water (containing HCl) and phenol. \square indicates material selected for refractionation. The qualitative compositions of the fractions (paper chromatography) are indicated diagrammatically above the curve.

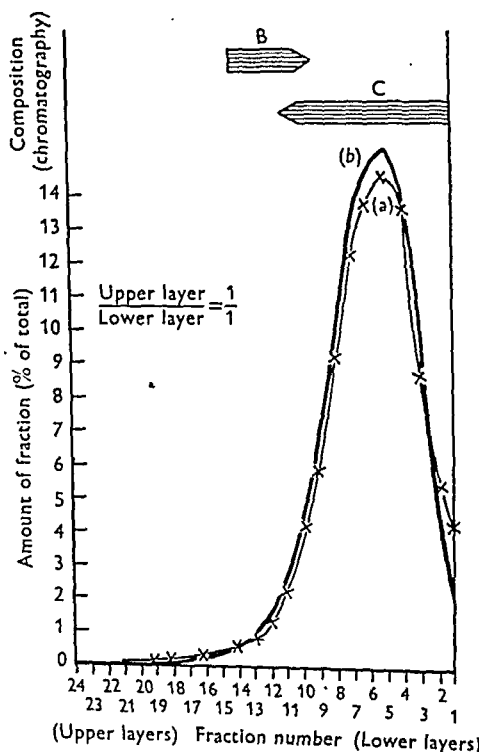


Fig. 5. Counter-current fractionation diagram for licheniformin C hydrochloride (a) partitioned between water (containing HCl) and phenol. A theoretical distribution curve (b) for $K=0.43$, $p=0.3$, is also shown. The qualitative compositions of the fractions are indicated diagrammatically above the curves.

curve, *b*, calculated on the assumption that $K=1.33$, is shown for comparison.

Generally, the procedure adopted was to select fractions rich in *B* from a number of different fractionations, unite them, fractionate with a ratio water/PhOH = 1/3 to remove the more water-soluble constituents, including *A*, to the upper layers, and then to unite the new fractions rich in *B* and conclude with a fractionation with a ratio water/PhOH = 4/3. The end result of one such fractionation is shown in curve *c* of Fig. 3.

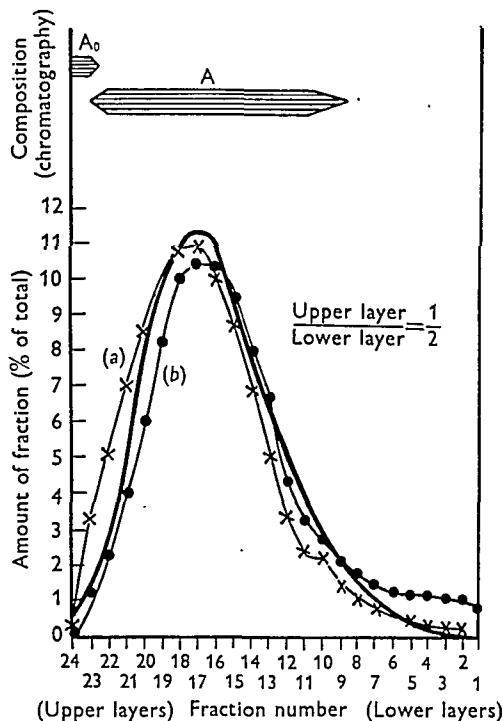


Fig. 6. Counter-current fractionation diagrams for two concentrates of licheniformin A hydrochloride partitioned between water (containing HCl) and phenol. A theoretical distribution curve for $K=3.0$, $p=0.6$ is also drawn (heavy line). The qualitative composition of the fractions (paper chromatography) is indicated diagrammatically above the curves.

Constituent *C* was isolated from a collection of *C*-rich fractions (wt. 3.8 g.) from various sources which, fractionated with a ratio water/PhOH = 1/0.5, gave the curve in Fig. 4. Fractions 6–12 from this (wt. 1.4 g.) were re-united and refractionated with a ratio water/PhOH = 1/1 with the result shown in Fig. 5, curve *a*. A theoretical curve *b* for $K=0.43$ has been drawn for comparison.

The same principles were applied to the separation of the constituent *A*. There was other material, A_0 , more soluble in water, and remaining at or very near to the origin of the paper chromatograms, from which *A* had to be separated. A_0 was a brownish, deliquescent material and there was no evidence of segregation into a fraction of uniform partition coefficient. The counter-current fractionation diagrams of two concentrates of constituent *A* are shown in curves *a* and *b* of Fig. 6, together with a theoretical curve for material of $K=3.0$.

Licheniformin mixtures from different sources

By comparison of the counter-current fractionation diagrams of crude licheniformin from the five different strains of *B. licheniformis* worked up by active charcoal or Decalso, no evidence was obtained that there was any considerable difference in the proportions of the three main constituents. Charcoal preparations contained less *A* and *B* and more material highly soluble in phenol in comparison with Decalso preparations. Another constituent appeared on one occasion. In fractions 4–9 of a counter-current distribution at water/PhOH = 1/3 of a crude product from A5, there was a constituent which moved slowly (at about the same rate as *a*) on a paper chromatogram. The amount of material available was not enough to encourage any attempt at isolation.

Some light was thrown on the composition of the material, often considerable in amount, at the extreme phenol-soluble end of the counter-current fractions by an experiment in which part of a lot of crude licheniformin was treated in 1% solution at pH 8–8.5 for 67 hr. at 37° with an equal weight of trypsin. The antibacterial potency was unaffected, and the only significant change in the counter-current diagram was a diminution of fraction 1 from 14% in the untreated material to 10% in the digested material. No effect on licheniformins A, B, or C was detectable.

PHYSICAL AND CHEMICAL PROPERTIES OF LICHENIFORMINS A, B AND C

Physical properties

All three constituents in the form of hydrochlorides were white, amorphous, slightly hygroscopic powders, melting with decomposition at indefinite temperatures. Each behaved as a single substance when analysed by paper chromatography using the collidine/lutidine/ammonia solvent mixture.

Optical rotation. Crude mixtures had $[\alpha]_D^{20} -36^\circ$ to -37.5° and $[\alpha]_{5461}^{20} -42^\circ$ to -47° (c, 1) in water. Purified licheniformin A hydrochloride had $[\alpha]_D^{20} -37.4^\circ$; $[\alpha]_{5461}^{20} -45.2^\circ$ in water (c, 0.19; l, 2 dm.). Licheniformin B hydrochloride had $[\alpha]_D^{20} -37.7^\circ$; $[\alpha]_{5461}^{20} -48.2^\circ$ in water (c, 0.84; l, 4 dm.). Licheniformin C hydrochloride had $[\alpha]_D^{20} -36.8^\circ$; $[\alpha]_{5461}^{20} -44.9^\circ$ in water (c, 1.18; l, 4 dm.).

Absorption spectra. The ultraviolet absorption spectra, measured on a Beckman spectrophotometer in aqueous solution, showed a rapidly increasing general absorption at decreasing wavelength below 2500 Å. Bands corresponding to phenylalanine or inflexions in this region were observed with preparations of all three constituents, most clearly with licheniformin B, least clearly with licheniformin C, which had the higher general absorption. Selected curves are given in Fig. 7. No firm conclusions could be based on these absorption spectra, for, apart from generally absorbing impurities, there was some evidence in one fractionation of a material with a partition coefficient between those of licheniformins B and C and not visible on paper chromatograms and which had an intense band at 2480 Å.

Molecular weight. We are indebted to Dr A. G. Ogston for determinations of molecular weights from sedimentation constants on the ultracentrifuge and diffusion constants. The values obtained were as follows: licheniformin A,

4400; licheniformin, B, 3800; licheniformin C, 4800. Full details are given in an Addendum to this paper (Addendum II).

Quantitative estimation of amino-acids in licheniformin: chemical method

Serine, glycine, proline, arginine and aspartic acid were estimated colorimetrically as their dinitrophenyl derivatives. A sample (40.3 mg.) of licheniformin A hydrochloride previously dried to constant weight at 100° was hydrolysed for 16 hr. in a sealed evacuated tube with excess 6N-HCl at 105°. The acid was removed and the residue dissolved in water and diluted to 4 ml. A sample of this solution (1.5 ml.) was allowed to react with an excess of fluorodinitrobenzene (FDNB) (0.15 ml.) in aqueous methanolic bicarbonate (6 ml. 5% (w/v) NaHCO₃, 6 ml. methanol) (Sanger, 1945) for 90 min. and the solution then acidified (10 ml. 3N-HCl).

From this point until all estimations were complete, operations were conducted entirely in a dimly lit room so as to avoid photodecomposition. The acid solution was extracted five times with 20 ml. peroxide-free ether, each extract being washed with 3 ml. water and the washings returned to the aqueous phase. Ether was removed from the aqueous phase under reduced pressure and the volume then adjusted to 50 ml. and 50 ml. of N-HCl added. Colour intensity was measured at once in a Hilger Spekker absorptiometer using a 1 cm. cell, tungsten lamp, violet filter (601) and heat filter (H 503). The amount of dinitrophenyl-(DNP-) arginine was determined by reference to a standard curve. The results are given in Table 1. Licheniformin B was treated in the same way. The combined ether extracts from the arginine determination were concentrated and the residue dried in a desiccator over KOH.

Use of Celite columns. Separation of the DNP-amino-acids was conducted on buffered Celite columns (Perrone, 1951). The Celite (Johns-Manville Co., London, S.W. 1), grade 545 (25 g.), was mixed with buffer (16 ml.) and peroxide-free wet ether (100 ml.) and stirred with a high-speed mechanical stirrer for 30 min. The suspension was poured into a suitable glass tube and the Celite packed with the packing tool described by Howard & Martin (1950). The diameter of the glass tube was chosen to give a final column about 15 cm. high. It was possible to separate from a single sample of 1.5 ml. of hydrolysed licheniformin hydrochloride (10 mg./ml.) the pure DNP derivatives of aspartic acid, serine, glycine, proline, valine and phenylalanine, but to do so it was necessary to use four successive columns of differing pH and the losses thus became serious. For quantitative estimation it was found that reproducible results could not be obtained unless the estimation of any one amino-acid was completed on a single column and within the compass of a single working day. The amino-acid which was being estimated was required to form a well defined band, separated from any other band, and the method of collection of the effluent was standardized both in this procedure and in the preparation of reference curves. When the band was 1 cm. from the bottom of the column the receiver was replaced by a measuring cylinder and when the trailing edge of the band left the column the volume of solvent in the cylinder was noted and a further 15 ml. of solvent collected before the receiver was changed. The solvent was evaporated as rapidly as possible *in vacuo*, the residue was dissolved in 1% NaHCO₃, the volume adjusted to 100 ml., and the colour intensity measured at once in an absorptiometer. By observing these precautions the variation between successive determinations of the same amino-acid was never greater than $\pm 5\%$.

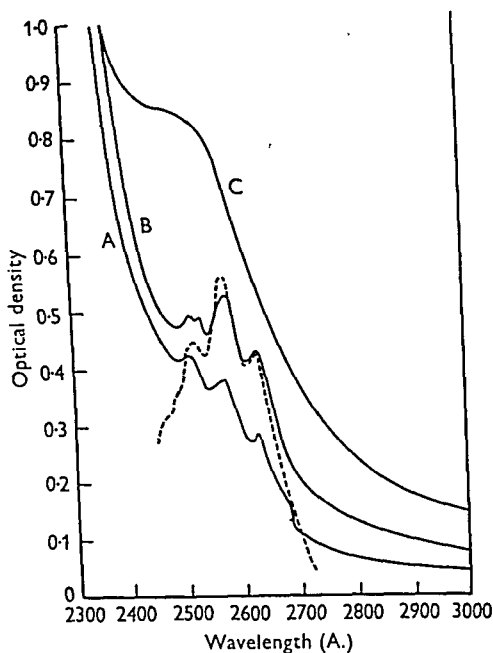


Fig. 7. Ultraviolet absorption spectra of licheniformins A, B and C and of phenylalanine (broken line). A and B were 0.2% (w/v) of hydrochloride in water, C was 0.1% (w/v) hydrochloride in water, and phenylalanine was 0.05% (w/v) in water.

Elementary analysis

The figures below are for material dried over P₂O₅ for 3-6 days.

Licheniformin	C (%)	H (%)	N (Dumas) (%)	Cl (%)
A	44.4	7.0	18.1	11.9
B (i)	44.7	7.2	18.4	10.4
B (ii)	46.2	7.1	17.7	11.3
C (i)	47.5	8.1	17.8	11.5
C (ii)	48.0	7.1	17.1, 16.4	—
C (iii)	47.5	7.7	18.1	—
C (iv)	45.2	7.0	17.0	10.5

(Analyses by Weiler & Strauss, Oxford, and by the Institute micro-analytical laboratory.)

Qualitative observation of peptide constitution

All three materials on acid hydrolysis gave amino-acids, evidently derived from an original peptide molecule. A sample of each antibiotic (250 µg.) was hydrolysed for 18 hr. with 6N-HCl at 105° in a sealed tube and after removal of acid the product was in each case analysed by two-dimensional paper partition chromatography. Licheniformins A and B gave chromatograms which indicated the presence of aspartic acid, serine, glycine, arginine, lysine, proline, valine and phenylalanine. Licheniformin C gave in addition a spot which was indistinguishable from glutamic acid.

Aspartic acid and serine. The Celite column was prepared in ether using buffer A of Blackburn (1949) and the ether then displaced by washed CHCl_3 . The ether-soluble DNP-amino-acids from 1.5 ml. of licheniformin solution (10 mg. licheniformin $\text{HCl}/\text{ml.}$) were dissolved in the minimum volume of washed CHCl_3 and transferred to the column. The column was developed with CHCl_3 until the two slow bands (aspartic acid and serine) were well separated; the fast bands were discarded. In order to speed development the CHCl_3 was replaced by CHCl_3 -butanol (99:1, v/v) and this solvent by CHCl_3 -butanol (98:2, v/v). The serine band was collected first and the aspartic band then pushed through with CHCl_3 -butanol (95:5, v/v). Estimations were made as described above. The results are given in Table 1.

Table 1. *Amino-acids in licheniformins A and B*

(Arginine, glycine, serine, proline and aspartic acid were estimated colorimetrically after conversion to their DNP derivatives and separation of the mixture on Celite. Phenylalanine, valine and lysine were estimated microbiologically.)

	Amino-acid (mg./100 mg. licheniformin)		N as % total N licheniformin	
	A	B	A	B
Aspartic acid	2.2	2.2	1.28	1.26
Glycine	11.9	11.8	12.3	11.9
Serine	6.5	6.9	4.8	5.0
Proline	5.5	5.5	3.7	3.6
Arginine	21.0	21.0	37.3	36.6
Phenylalanine	8.3	9.0	3.9	4.2
Valine	4.8	5.5	3.2	3.6
Lysine	36.6	38.6	38.6	40.0
Total	—	—	105.08	106.16

Glycine and proline. The procedure was the same as that used for aspartic acid and serine except that ether was used as solvent and the column was buffered with a mixture of 0.5M- KH_2PO_4 (975 ml.) and 0.5M- Na_2HPO_4 (25 ml.). The fast bands ($R \rightarrow 1$) were discarded and the next two bands (proline and glycine) collected. The results are given in Table 1.

Valine, lysine and phenylalanine. No column could be devised which would separate these three amino-acids as their DNP derivatives in a manner which eliminated all danger of error from overlapping bands and we accordingly abandoned the method in favour of microbiological assay.

Synthetic mixture. A mixture of glycine (25 mg.), serine (25 mg.), valine (25 mg.), phenylalanine (25 mg.), aspartic acid (25 mg.), arginine (75 mg.), and lysine (150 mg. monohydrochloride, $2\text{H}_2\text{O}$) in water was diluted to 15 ml. and samples of 0.5, 1 and 2 ml. used for preparation of the DNP derivatives. Three successive determinations were made of colour intensity for arginine, aspartic acid, serine, glycine and proline at each of these levels, the procedure outlined above being followed in each case. The results were highly reproducible, e.g. scale reading on absorptiometer: 0.475, 0.475, 0.485 for DNP-arginine from 2 ml. solution, and 0.25, 0.27, 0.26 for DNP-arginine from 1 ml. solution; 0.56, 0.58, 0.57 for proline from 2 ml. solution, and 0.38, 0.38, 0.39 for proline from 1 ml. solution.

*Quantitative amino-acid analyses:
microbiological methods* (Miss K. R. de Bouk)

An account of these is given in an Addendum to this paper (p. 567).

D-Amino-acids in licheniformins A and B

A preparation of D-amino-acid oxidase was made from sheep kidney by the method of Negelein & Brömel (1939). Purification was carried only to the stage of the first $(\text{NH}_4)_2\text{SO}_4$ precipitation. Samples of the peptides were

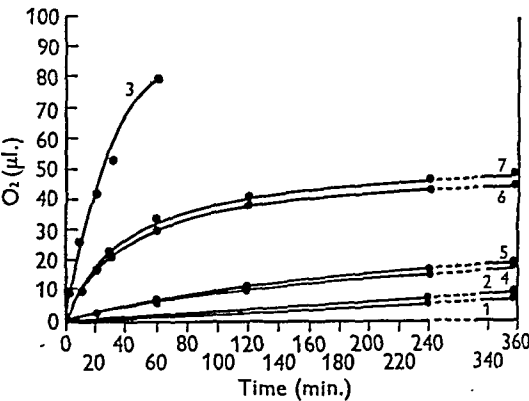


Fig. 8. Oxygen uptake (in $\mu\text{l.}$ at 37°) during oxidation of hydrolysed licheniformin by D-amino-acid oxidase (for conditions see text). Curve 1, no substrate; curve 2, L-phenylalanine; curve 3, D-phenylalanine; curve 4, hydrolysed horse serum globulin; curve 5, hydrolysed casein; curve 6, licheniformin A; curve 7, licheniformin B.

hydrolysed for 8 and 16 hr. at 100° in 6N-HCl in sealed, evacuated tubes. As controls, samples of casein and horse serum globulin were hydrolysed in the same way. The acid was removed from each sample by leaving in a desiccator over KOH for several days and the residue dissolved in water. The pH was adjusted to 8.0 by addition of 5% NaHCO_3 and the solution diluted to a concentration equivalent to 10 mg. peptide or protein/ml. The amount of D-amino-acid in each preparation was estimated by following O_2 uptake in Warburg manometers. Each flask contained 0.5 ml. hydrolysed peptide, 1.8 ml. sodium pyrophosphate buffer (pH 8.3, 0.067M) and, in the centre cup, 0.2 ml. KOH (20%, w/v). The side arm of each Warburg flask contained 0.2 ml. enzyme. As controls three flasks were prepared, one with no substrate, one with D-phenylalanine (0.5 ml. \equiv 2.5 mg. amino-acid) and one with L-phenylalanine (0.5 ml. \equiv 2.5 mg. amino-acid). The result of a typical experiment is shown in Fig. 8.

Three successive runs were performed on separate samples of each peptide hydrolysed for 8 hr. and one on each peptide hydrolysed for 16 hr. The results were the same within the limits of error of the method. The two proteins hydrolysed for 8 hr. and for 16 hr. showed a slightly greater O_2 uptake than the control or the L-phenylalanine (10 $\mu\text{l.}$ in 360 min.). After subtraction of the control value the corrected O_2 -uptake figures for licheniformin A were 40, 37, 35, and 45 $\mu\text{l. O}_2/5$ mg. peptide hydrochloride, and for licheniformin B 45, 39, 35, and 45 $\mu\text{l. O}_2/5$ mg. peptide hydrochloride. These figures indicate that each peptide contained the same proportion of amino-acid in the D form. In the hope that the

D-amino-acid could be identified, the material from each Warburg flask was freed from enzyme (heat coagulation) and a portion was used for two-dimensional paper partition chromatography. Both peptides had initially given eight different amino-acids on hydrolysis and the same eight amino-acids were still present after oxidation with D-amino-acid oxidase. The conclusion is that in each peptide at least one species of amino-acid is present in both optical forms.

End amino groups in licheniformins A and B

The method of Sanger (1945) for the estimation of end groups using fluorodinitrobenzene was applied to each of the peptides. Each peptide (25 mg.) was treated with FDNB in the usual way and, after hydrolysis for 24 hr. at 105° with 10N-HCl (sealed tube), the product was separated into a water-soluble and an ethyl acetate-soluble fraction. The ethyl acetate-soluble fraction was passed through a column of silica gel and the very faint yellow band collected. Measurement of the colour intensity of this band in each case showed that it represented less than 6% of the theoretical value required for one end group. A sample of each DNP-peptide was also hydrolysed for 6 hr. with 6N-HCl, but no end group was found. Confirmation that both peptides had failed to react with FDNB was obtained by paper chromatography of the water-soluble fraction from the acid hydrolysis. This fraction contained ϵ -dinitrophenyllsine, and in addition, all the other amino-acids present in a simple acid hydrolysate of the peptide. The DNP-lysine was put on a column of silica with methyl ethyl ketone-ether (Sanger, 1945) as solvent. A single band indistinguishable from synthetic ϵ -DNP-lysine was obtained.

BIOLOGICAL TESTS

Antibacterial activity. The three antibiotics were tested *in vitro* against *Mycobact. phlei* and the greatest dilution capable of inhibiting surface growth in Hartley digest broth was recorded. Licheniformin A (hydrochloride) inhibited at 1 in 5×10^6 ; licheniformin B at 1 in 10×10^6 ; and licheniformin C at 1 in 0.32×10^6 . The figures quoted are averages for three successive tests.

Chronic toxicity (mouse). Table 2 shows the results of chronic toxicity tests for which we are indebted to Dr Janet Niren. The antibiotics were made up in sterile saline at about 5 mg./ml. and injected subcutaneously in mice. A sample of unfractionated licheniformin corresponding in properties with the licheniformin hydrochloride used in previous toxicity tests (Callow *et al.* 1947) was used for comparison and a sample of bacitracin was also tested. The assessment of kidney damage is subjective, but was always made by the same person and indicates the degree of micro-anatomical alteration in the kidneys of the experimental animals.

Mouse protection test against tuberculous infection. As licheniformin A was found by the chronic toxicity test to be much less toxic than B or C, it was tested further for its ability to control tuberculous infection in mice. The tests were made by the procedure outlined in an earlier paper (Callow *et al.* 1947) and streptomycin was simultaneously tested as a control. We are indebted to Dr P. D'Arcy Hart and Dr R. J. W. Rees for carrying out the tests. Licheniformin A was tested at two dose levels, 3 mg./day and 6 mg./day. The lower dose caused no obvious toxic symptoms but failed to control the infection; the higher dose controlled the infection to a lesser degree than streptomycin at the level of 0.3 mg./day and caused some kidney damage.

Table 2. *Chronic toxicity for mice of licheniformins A, B and C, compared with standard preparation and with bacitracin*

(Licheniformin A hydrochloride 5 mg./ml., licheniformin B hydrochloride 5 mg./ml., licheniformin C hydrochloride 7 mg./ml., bacitracin* 5 mg./ml., licheniformin standard (S 785) 5 mg./ml. Dose schedule 0.2 ml. morning, 0.4 ml. evening, and 0.4 ml. Saturday for 14 days (no dose given on Sundays). Each test made on three animals. The degree of kidney damage is indicated; +, slight; + + +, severe.)

Licheniformin			Bacitracin	S 785	Control (saline)
A	B	C			
+	+	+	+	+	—
+	+	+	+	+	—
+	+	+	+	+	—
			(died)		

* A specimen of commercially manufactured product provided by Dr F. L. Meleney.

DISCUSSION

A large number of antibiotics have been reported in the culture filtrates from aerobic spore-forming bacilli (Baron, 1950). The nature of the substance responsible for antibiotic action is not known in every case, but where a pure or nearly pure product has been isolated, activity has frequently been found in a peptide fraction (Gilliver, Holmes & Abraham, 1949; Sharp, Arriagada, Newton & Abraham, 1949; Howell, 1950; Catch, Jones & Wilkinson, 1949; Barry, Gregory & Craig, 1948; Consden, Gordon, Martin & Synge, 1947; Hotchkiss, 1944). In some instances an antibiotic peptide, which was at first thought to be homogeneous, has later been resolved into several distinct substances (Gregory & Craig, 1948).

In the present investigation, numerous attempts at purification of licheniformin hydrochloride by precipitation, salt formation and adsorption chromatography gave little or no evidence of heterogeneity, but paper partition chromatography showed that the best preparation of licheniformin previously obtained (Callow *et al.* 1947) was a mixture of at least three antibiotics. Counter-current distribution methods (Bush & Densen, 1948; Craig & Post, 1949) were successful in separating these three antibiotics, but nevertheless the purity of the final products, the hydrochlorides of licheniformins A, B and C, cannot be regarded as absolutely established. Each substance gave a single zone of colour with ninhydrin after paper chromatography and this zone coincided entirely with the zone of antibiotic activity. Each substance gave a distribution curve in a 24-stage counter-current fractionation which followed closely the theoretical curve for a pure compound. The materials are, therefore, each homogeneous with respect to partition coefficient between two pairs of solvents, but no crystalline derivative could be obtained.

We have been informed that Dr L. H. Kent, Dr B. T. Tozer and Dr J. H. R. Slade at the Microbiological Research Department have obtained very similar results with products from the A5 strain on ammonium lactate medium. Like ourselves, they would put forward only with the greatest reserve the statement that any fractions are chemically homogeneous.

Biological activity

Crude licheniformin hydrochloride (Callow *et al.* 1947) had a wide antibacterial spectrum, but its main interest lay in its considerable activity against acid-fast organisms and in its potentialities as an antitubercular drug. The limitation to the use of the crude antibiotic was that it caused severe damage to the kidney. Purified licheniformin C was less active against acid-fast organisms than the original crude preparation and caused more extensive kidney damage. Licheniformin B was rather more active *in vitro* than the original preparation, but it also caused extensive kidney damage. Licheniformin A was much less toxic than either of the other fractions, but still caused some slight kidney damage and was less effective than streptomycin in controlling tuberculosis in mice (p. 565). It would be tempting to suppose that renal toxicity is not an inherent property of licheniformin A and that the small degree of kidney damage evident in our tests was due to contamination of this substance with another peptide but, since the criteria of purity were fairly exacting, we do not ourselves favour this view. Renal toxicity has been reported also with several other antibiotic peptides obtained from related bacteria, and attempts to separate this toxicity from the antibacterial activity have not been entirely successful (Miller, McDonald & Shock, 1950; Smith, Schultz, Ott & Payne, 1949; Abraham & Newton, 1950).

Chemical structure

The difficulty experienced in separating the three compounds indicated some similarity of composition, but we did not expect such close similarity as was actually found. Licheniformin C was readily distinguishable from the other two peptides in that it gave, on acid hydrolysis, glutamic acid in addition to the eight amino-acids found in licheniformins A and B. In other respects the three compounds showed remarkable similarity. Elementary analyses did not distinguish between them; all three had the same optical activity and about the same molecular weight; all three, after reaction with fluorodinitrobenzene, failed to give any 'end group' (Sanger, 1945) on acid hydrolysis.

Licheniformins A and B, being the more active

antibiotics, were examined in greater detail than C. As can be seen from Table 1, the amounts of amino-acids produced on hydrolysis are strikingly similar: they account quite well for the whole of the original peptides and it seems unlikely that the difference in toxicity between A and B could be due to some unrecognized non-peptide component. The difference between the molecular weights of licheniformins A and B (4400 and 3800) is scarcely significant, having regard to the errors of the method, and in Table 3 the number of amino-acid residues/molecule of peptide is calculated on the assumption that the mean of these figures is the molecular weight of both peptides. There are three major sources of error in this calculation: (i) molecular weight, (ii) percentage composition as determined by micro-analyses and (iii) estimation of individual amino-acids by colorimetric or microbiological methods. Tristram (1950) has pointed out that in order to estimate the stoichiometric ratio of amino-acids in a protein or peptide the percentage error in the analysis of any one amino-acid must not exceed $\pm 0.4/R \times 100$, where R is the number of residues of that amino-acid. We have not had sufficient material to permit repeated quantitative analyses on our antibiotics, but we consider that the methods used cannot be relied upon to be more accurate than $\pm 5\%$, thus it may well be that the error approaches 10% with a few of our amino-acid estimations.

Table 3. *Amino-acid residues in licheniformins A and B*

(Results are calculated assuming a molecular weight of 4100 for each peptide. Figures are given to nearest whole number but where there is doubt, the calculated analytical figure is given in brackets. For definition of permissible error, see text.)

	Licheniformin A	Licheniformin B	Permissible error (%)
Aspartic acid	1	1	40
Glycine	7 (7.4)	7 (7.2)	5
Serine	3	3	13
Proline	2	2	20
Arginine	6 (5.65)	6 (5.5)	6.7
Phenylalanine	2 (2.4)	2 (2.5)	13
Valine	2	2	20
Lysine	12	12	2.8

The last column of Table 3 gives the percentage accuracy required to calculate, with certainty, the number of residues of each amino-acid present in the unit of molecular weight 4100. It is apparent from these figures that our failure to find any significant difference in residue numbers between the two peptides does not necessarily prove that they have the same quantitative composition.

With these reservations in mind the close chemical resemblance between licheniformins A and B is

nevertheless quite striking and the difference in toxicity is difficult to explain. A difference in optical form of the constituent amino-acids of these two peptides is not attractive as an explanation, since each peptide, after hydrolysis, gave an amino-acid mixture in which the same proportion of amino-acid was present in the D form; however, the species of D-amino-acid was not established in either case. We are more inclined to suggest that the difference in toxicity between licheniformins A and B may be attributable to a difference in amino-acid sequence within the two peptides. Some attempts were made to confirm this idea by partial hydrolysis experiments, but both compounds gave mixtures of basic peptides which we could not fractionate.

The suggestion has already been made (Work, 1949) that certain antibiotics may act by interference with protein synthesis. It was pointed out that protein synthesis is a species-specific process and that inhibition of protein synthesis by metabolite analogues might be expected to be species-specific also. We have now obtained some evidence (Campbell & Work, 1951) that peptides are normal intermediates in protein synthesis and it is therefore not surprising that amino-acid sequence in an antibiotic peptide may influence toxicity. We have, as yet, no clue to the cause of the common nephrotoxic action of antibiotic peptides.

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1. The licheniformin of Callow *et al.* (1947) has been separated by the use of counter-current distribution into three antibiotic peptides, licheniformins A, B and C.

2. Licheniformins A and B are both hydrolysed by acid to give glycine, serine, proline, valine, phenylalanine, lysine, arginine and aspartic acid. Licheniformin C gives these amino-acids and, in addition, glutamic acid.

3. All three peptides have similar molecular weights (3800–4800), optical rotations and elementary compositions.

4. Licheniformins A and B are more active against *Mycobacterium phlei* than licheniformin C, and are less toxic to mice than licheniformin C.

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ADDENDUM I

Microbiological Estimation of Lysine, Valine and Phenylalanine in Licheniformin

By KATHERINE R. DE BOUK

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(Received 10 October 1951)

Although the estimation of lysine, valine and phenylalanine by microbiological methods is a standard procedure, some difficulty was experienced in using, unmodified, the media of earlier authors. After testing the media of Dunn, Shankman, Camien, Frankl & Rockland (1944), Barton-Wright (1946), Sauberlich & Baumann (1946) and Steele, Sauberlich, Reynolds & Baumann (1949), some modifications were made to the last two, and good acid production with linearity over a substantial part of the curve was attained. A brief account of these modifications is given. The results obtained are given in Table 1 (p. 564).

MATERIALS AND METHODS

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Stock stab cultures were carried in a Nymon & Gortner (1946) medium, modified as follows. For 100 ml. of medium: Difco bacto-peptone, tryptone and yeast extract, 0.5 g. each; liver extract, 10 ml.; glucose 1.0 g.; sodium acetate (hydrated), 1.0 g.; 0.5 ml. each of salt solutions A and B (see basal medium); agar, 1.5 g. This medium maintained vigorous growth for all the organisms commonly used for amino-acid assays (*Lb. arabinosus* 17-5, *Lb. mesenteroides* P-60, *Streptococcus faecalis* R, *Lactobacillus fermenti* 36

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Leuconostoc citrovorum, *Lactobacillus brevis*), and was an improvement on published media on which cultures tended to die out. Transfers were made monthly. This practice was discontinued, however, in the case of *Lb. arabinosus* owing to a gradual departure in linearity of response and the latter was maintained in the dried state. Before use in assays the organisms were grown in broth of the same composition as the above, with the agar omitted.

Basal medium. Medium I of Sauberlich & Baumann (1946) was used with *Lb. arabinosus* 17-5 with the following modifications. Serine, glycine and proline were omitted from the medium as unnecessary; sodium acetate (hydrated) was increased from 20 to 33 g./500 ml. of double-strength medium; 200 mg. of L-asparagine was replaced by 640 mg. of DL-aspartic acid; riboflavin was reduced from 0.5 to 0.25 mg. (see below).

Medium VI of Steele *et al.* (1949) used with *L. mesenteroides* P-60 was modified by increasing the vitamins. 500 ml. of double-strength medium contained: aneurin 1.0 mg.; pyridoxin, 1.6 mg.; calcium D-pantothenate, 1.0 mg.; nicotinic acid, 2.0 mg.; p-aminobenzoic acid, 0.1 mg.; biotin, 0.005 mg.; folic acid, 0.01 mg.; riboflavin, 0.25 mg. The smaller quantity of riboflavin used in both media I and VI served to diminish the colour of the medium and hence to facilitate titration, and in no way affected the assays.

The inorganic salt solutions used to make up 500 ml. of media (double strength) were as follows: Solution A,

500 mg. of KH_2PO_4 and 500 mg. of K_2HPO_4 in 5 ml. Solution B, 200 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, and 10 mg. of NaCl in 5 ml.

Standard curve. The synthetic DL-acid was used, after confirming that the D-form was not available with the media employed; however, calculations were based on the L-isomers only. The range (per tube) for the construction of the linear portion of the curve was as follows: L-lysine HCl, 0-50 μg ., L-valine and L-phenylalanine, 0-25 μg .

Preparation of samples for assay. Licheniformin hydrochloride (15 mg.) was hydrolysed in 6N-HCl for 16 hr. at 105° in a sealed evacuated tube. The HCl was removed over KOH and the residue made up to 10 ml. in water. Portions taken were adjusted to pH 6.8.

Assay procedure. The sample was added to 5 ml. quantities of double-strength medium and the tubes sterilized at 10 lb./in.² for 5 min. This gave less darkening of the medium than with the longer periods and higher pressures commonly used. The lactic acid was titrated to pH 6.8 with 0.1N-NaOH using Smith's (1930) indicator suitably altered so as to produce the colour change of orange-grey to pure grey at pH 6.8 instead of 6.9. This modified indicator, containing 0.09 g. of bromothymol blue and 0.10 g. of alizarin red sulphonate dissolved in 100 ml. of 30% (v/v) aqueous ethanol, gives a much sharper end point than the bromothymol blue always recommended in the literature for microbiological assay titrations.

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ADDENDUM II

Sedimentation and Diffusion of Licheniformins A, B and C

By A. G. OGSTON

Department of Biochemistry, University of Oxford

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Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948). The hydrochlorides were dissolved in 0.2M-NaCl to a concentration of 1 g./100 ml. Owing to the relatively small molecular weights and high diffusion coefficients, clear sedimentation boundaries were not obtained; the sedimentation constants were therefore measured by the method of Gutfreund & Ogston (1949). These results are probably not more accurate than $\pm 5\%$.

Diffusion. This was measured in the Gouy diffusimeter (Coulson, Cox, Ogston & Philpot, 1948). The same solutions were used as for sedimentation. Since an equilibrium diffusate could not be prepared by dialysis, the solvent into which diffusion took place was NaCl of a concentration

calculated to equal that of diffusate on the basis of the Gibbs-Donnan equilibrium. The observed diffusion coefficients are then comparable with the sedimentation constants. Both sedimentation and diffusion constants are corrected to their values in water at 20° (Table 1). In calculating the molecular weights, a value of 0.75 has been assumed for the partial specific volume.

Table 1. *Sedimentation and diffusion constants*

Licheniformin	$S_{20} \text{ (corr.)} \times 10^{13}$	$D_{20} \text{ (corr.)} \times 10^7$	Mol.wt.
A	0.96	21.0	4400
B	0.86	22.4	3800
C	0.95	19.4	4800

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A Solubility Analysis of Crystalline Ox-Liver Catalase

By G. L. BROWN

Medical Research Council Biophysics Unit, Department of Physics, King's College, Strand, W.C. 2

(Received 27 October 1951)

Loew (1901) first demonstrated that the decomposition of hydrogen peroxide by plant and animal tissues was due to an enzyme which he named catalase. Other workers proceeded to isolate and purify this enzyme and eventually Zeile & Hellström (1930) established that the enzyme was an iron-porphyrin protein. This was confirmed by Keilin & Hartree (1936) and Stern (1935), who also proved that the iron-porphyrin was protohaematin. Sumner & Dounce (1937) crystallized ox-liver catalase soon afterwards; since then many other liver and erythrocyte catalases and a bacterial catalase have also been prepared in a crystalline state.

Catalase has been found to be present in all aerobic cells so far examined and is present in high concentrations of from 10^{-6} to 10^{-7} M in the liver,

kidneys and erythrocytes of mammals. Dounce (1943) has stated that the catalase of liver is confined to the cytoplasm of liver cells, but has since found (1951) that this observation was due to the method of preparation of the liver-cell nuclei and that nuclei prepared by an improved method do contain catalase.

Stern & Wyckoff (1938), using an ultracentrifuge method, found the molecular weight of ox- and horse-liver catalases to be about 250 000, and from the iron content of their preparations calculated that the catalase molecule contained four atoms of iron. Sumner & Dounce (1937) observed that only about half the iron of their crystalline ox-liver catalase preparations was bound as haematin, and Lemberg, Norrie & Legge (1939) later demonstrated

that part of the iron of liver catalases is bound in the form of a catalytically inactive bile-pigment haematin. Bonnichsen (1948) has put forward evidence which indicates that the bile-pigment content of liver catalases may be due to the methods of preparation.

The fractional values for the molecular ratio of haematin and catalase protein and the variations of specific activity obtained with different preparations, has prompted Sumner (1941) to suggest that liver-catalase preparations contain a mixture of catalases with one, two, three and four intact haematin molecules per catalase molecule, the remaining iron being bound as inactive bile-pigment haematin. According to Lemberg & Legge (1949), the values of specific activity or catalase capability (*Kat.f.* defined by von Euler & Josephson, 1927) for most purified preparations of liver catalases described in the literature vary between 52 000 and 60 000, when corrected to zero bile-pigment content. Agner (1942), however, has reported a *Kat.f.* value of 80 000 for a horse-liver catalase preparation, when corrected to zero bile-pigment content. This value has since been confirmed by Agner & Theorell (1946).

In a preliminary study of the Sumner & Dounce method of preparing ox-liver catalase, the author found that homogenizing the minced tissue in a Waring Blendor increased the measured *Kat.f.* of the crystalline catalase from 45 000 to 72 000. The present paper describes an analysis of this highly active preparation using a solubility test method designed to detect and characterize the components of the suspected mixture of catalases. It is shown that the increase in *Kat.f.* of the final product after homogenizing the tissue is due to the presence of a small amount of a catalase with a much higher *Kat.f.* than hitherto observed, and some of the optical properties of this new catalase have been measured.

EXPERIMENTAL

Preparation of enzyme

Crystalline ox-liver catalase was prepared by a modification of the method of Sumner & Dounce (1937) as follows:

Ox liver (2 kg.) was minced, homogenized for 2 min. at 0° in a Waring Blendor, extracted with 35% (v/v) aqueous dioxan and fractionated with dioxan at 0° according to Sumner & Dounce. The precipitated fraction, which contained the catalase, was extracted three times with a dilute solution of salivary amylase in distilled water (total vol. 25 ml.) to digest the glycogen, and the catalase isolated from this extraction solution in a crystalline form by seeding and allowing to stand at 0° for 24 hr. The crystals were centrifuged down at 0°, dissolved in 0.1 M-phosphate buffer, pH 7.4, and recrystallized by dialysing against 0.1 M-phosphate buffer, pH 6.3. These crystals were centrifuged at 0°, dissolved in 0.1 M-phosphate buffer, pH 7.4, and dialysed against 0.1 M-phosphate buffer, pH 6.55. The mother

liquors from the two crystallizations were also dialysed against this buffer for 2 days before carrying out the solubility test.

Solubility test method

Amounts of $(\text{NH}_4)_2\text{SO}_4$ calculated to produce a suitable range of ionic strengths were dissolved in 5 ml. samples of the enzyme solutions containing about 1 mg. protein/ml. After allowing to equilibrate at 0.5° for 24 hr. the solutions were filtered at the same temperature, as described by Falconer & Taylor (1946*a*). The optical densities of the filtrates at 275, 405 and 502 m μ . the maxima of the catalase absorption spectrum, were measured in a Beckman spectrophotometer. This procedure was carried out with the twice-crystallized material and with the two mother liquors. The *Kat.f.*, of the twice-crystallized catalase, was determined by the method of von Euler & Josephson (1927) and the relative catalase activities of the filtrates determined by the perborate method of Feinstein (1949) at 10°. The protein N content of some of the filtrates, selected on the basis of the salting-out curves, were estimated by the micro-Kjeldahl method of Falconer & Taylor (1946*a*). Total haematin content was estimated by the method of Duve (1949).

RESULTS

The salting-out curves for the twice-crystallized material in terms of the variations of optical densities at 275, 405 and 502 m μ . with ionic strength are shown in Fig. 1*a-c* respectively, and the corresponding changes in enzyme activity are shown in Fig. 1*d*. The specific property solubility diagrams of Falconer & Taylor (1946*b*), shown in Fig. 1*e, f*, clearly demonstrate the complex nature of the preparation. It is apparent from Fig. 1 that there are three main components *A, B* and *C* with appreciable absorption coefficients at 405 m μ ., together with a small amount of material that absorbs at 275 m μ . but not at 405 m μ . and remains in solution at an ionic strength of 6.5. An analysis of the constitution of the preparation and of the nature of the components, derived from the solubility curves and from the protein-nitrogen estimations, is presented in Table 1.

Protein *C* has a *Kat.f.* value of 45 000 and a ratio of the optical density at 405 m μ . to that at 275 m μ . of 0.9 and therefore corresponds to the catalase usually obtained from ox liver by the unmodified Sumner & Dounce method. From the complexity of the sections of the graphs in Fig. 1*e, f*, corresponding to the salting-out of this component, it is possible that *C* is a mixture of catalases differing only in the number of intact haematin molecules per catalase molecule. Protein *A*, with a *Kat.f.* value of 180 000, has not been observed before, and its presence accounts for the increase in *Kat.f.* to 72 000, when the usual method is modified by the introduction of the blending process. The *B* component is apparently a haem protein with no appreciable catalase activity.

The observation of the optical densities of those filtrates of the two mother liquors with ionic strengths between 3.5 and 5.2 was prevented by the following interesting effect. Immediately a portion

cloudiness disappeared, but reappeared again on exposure to the radiation from the Beckman or to ordinary daylight. No appreciable change in temperature occurred in the solution during this

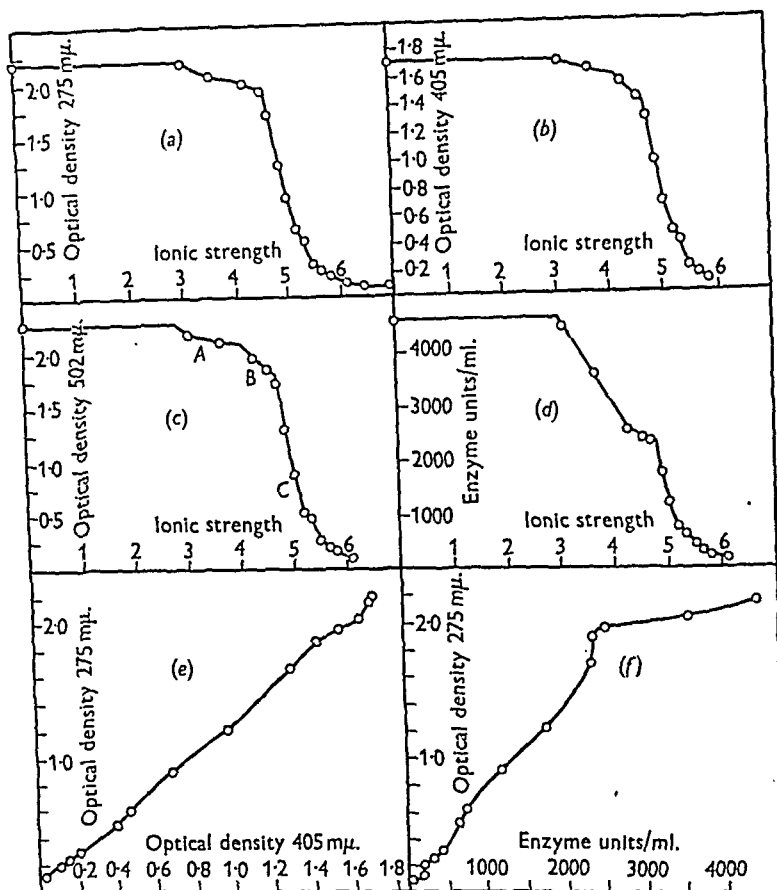


Fig. 1. Solubility test curves of twice-crystallized ox-liver catalase at pH 6.55 using solid $(\text{NH}_4)_2\text{SO}_4$ as salting-out agent. (a), (b) and (c) are the precipitation curves for proteins in solution with absorption at 275, 405 and 502 $\text{m}\mu$. respectively; (d) is the precipitation curve for catalases in solution, and (e) and (f) are the 'specific property solubility curves' for the solution.

Table 1. Solubility test analysis of twice-crystallized ox-liver catalase constitution of original solution

(Kat.f.=72000; moles haematin/moles catalase=3.2; optical density 275 $\text{m}\mu$ /optical density 405 $\text{m}\mu$.=1.3; % component A=17; % component B=4; % component C=78.)

Properties of the components

	A	B	C
Enzyme activity/optical density 275 $\text{m}\mu$.	6800	0	1810
Enzyme activity/optical density 405 $\text{m}\mu$.	15490	0	1630
Optical density 405 $\text{m}\mu$ /optical density 275 $\text{m}\mu$.	0.43	1.3	0.90
Optical density 502 $\text{m}\mu$ /optical density 275 $\text{m}\mu$.	0.048	0.13	0.11
Optical density 502 $\text{m}\mu$ /optical density 405 $\text{m}\mu$.	0.11	0.10	0.12
Kat.f.	180000	0	45000

of these filtrates was exposed to radiation of wavelengths 275, 405 or 502 $\text{m}\mu$. issuing from the monochromator of the Beckman spectrophotometer, the clear solution became cloudy due to precipitation. After placing the samples in darkness for 1 hr. the

precipitation process, and the effect could not be produced by raising the temperature of the samples to 30°. Fig. 2, which shows the salting-out curve of the mother liquor from the second crystallization, indicates that a component (X) is being salted out

over the range of ionic strengths at which the 'photo-precipitation' effect occurs and therefore that component *X* is in equilibrium with its solvent in the filtrates with ionic strengths in this range. It seems clear, then, that the precipitation effect is due to a decrease in solubility of this component on absorption of visible and ultraviolet radiation. Its salting-out properties, and the fact that it contains

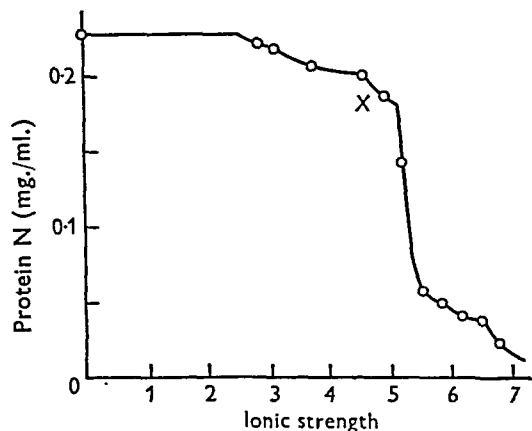


Fig. 2. Precipitation curve of mother liquor from the second crystallization of ox-liver catalase showing the precipitation of a protein *X* in the range of ionic strengths over which a 'photo-precipitation' effect occurs when the solutions are irradiated with light.

nitrogen, and its existence in a solution of a crystallized protein indicate the protein nature of component *X*. Apparently, it is left entirely in the mother liquor after crystallization at pH 6.3, as the precipitation effect was not observed in the solubility test on the twice-crystallized material.

DISCUSSION

The existence of component *A* in the preparation analysed above is undoubtedly due to the action of the Waring Blender, which disintegrates the tissue much more than mincing. This indicates that *A* is strongly bound to insoluble particles in the liver cells, only being released into solution when these are damaged by the blending process. This explanation is supported by the observation of Falconer & Taylor (1946*a*) that catalase activity is released from thoroughly washed liver mince after autolysis, and by the work of Still & Kaplan (1950), which showed that the action of the Waring Blender could bring about the transfer of the glycolytic oxidase activity of a suspension of mitochondria from the particulate elements into solution.

The value of 0.43 for the ratio of the optical density at 405 m μ . to that at 275 m μ . for component *A*, indicates that *A* is distinctly different from *C* for which the corresponding ratio is 0.90. These values suggest that either component *A* has

a much lower ratio of haematin to protein molecules than *C* or that its protein component has a higher aromatic amino-acid content than the *C* protein. The latter is identical in *Kat.f.* and optical properties with the catalase preparations obtained without the use of the blender and is therefore unbound, or not so firmly bound, to insoluble particles in the cells as the *A* component. In view of the above facts it seems probable that the *C* component is partially degraded erythrocyte catalase, with lowered specific activity due to its content of bile pigment haematin, which is on the pathway of degradation of haematin in liver. This explanation would account for the ease with which it can be extracted and its low activity. The *A* component, however, would appear to be the 'structural' catalase of the liver in view of its high specific activity and the drastic treatment needed for its extraction.

By fractional crystallization of crystalline horse-erythrocyte catalase prepared according to Bonnichsen (1947), Deutsch (1951) has recently obtained preparations with *Kat.f.* values ranging from 84 000 to 118 000, but with identical absorption properties. The high-activity fraction was found to be extremely labile, the *Kat.f.* value decreasing to 84 000 on standing at 0° or on dilution, without any change in its absorption properties. The two components observed in the ox-liver catalase preparation described in this paper have different optical properties, and the highly active component appears in the preparation only after homogenization. This would seem to preclude any relationship between them such as that observed by Deutsch in the case of erythrocyte catalase preparations.

SUMMARY

1. A modification of the Sumner & Dounce method of preparing crystalline ox-liver catalase has been developed which yields a product with a *Kat.f.* of 72 000.

2. It has been demonstrated by a solubility test technique that the product contains two distinct catalases with *Kat.f.* values of 45 000 and 180 000.

3. It is suggested that the catalase with the *Kat.f.* value of 180 000 is attached to insoluble complexes in the liver cell and is the structural catalase of liver.

4. A protein exhibiting a reversible change in solubility on exposure to radiation of wavelengths in the visible and ultraviolet regions of the spectrum has been detected in partially purified catalase preparations.

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Effects of Cold Environment on Deposition of Fat in the Liver in Choline Deficiency

By E. A. SELLERS AND ROSEMARY W. YOU
Department of Physiology, University of Toronto

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In a preliminary communication (Sellers & You, 1949) it was reported that when rats were exposed to a cold environment the deposition of excess fat in the liver produced by feeding a hypolipotropic diet was effectively prevented. Only one set of experimental conditions had been tested, so that it was of interest to extend the investigation by varying the length of exposure and the type of diet offered, and to study the effects of acclimatization in relation to the phenomenon. Our interest in the effects of the thyroid gland on deposition of fat in the liver (Sellers & You, 1951) also made it desirable to assess the role of this gland in the 'pseudolipotropic' action of cold.

EXPERIMENTAL

In all experiments rats of the Wistar strain, bred in the department, were kept in individual metal cages and were given water *ad lib.* After the experimental period they were killed by cervical dislocation and the livers were removed for chemical and histological examination. Total liver lipids were estimated using the method of Best, Lucas, Patterson & Ridout (1946). The basal diet consisted of peanut meal 30%, casein 6%, salts 4% (Beveridge & Lucas, 1945), sucrose 39%, 'vitamin powder' 1%, beef fat 15%, corn oil 5%, α -tocopherol acetate 0.010%, cod-liver oil concentrate 0.015%. The 'vitamin powder' was of such a composition that the intake per 10 g. of diet was as follows: Biotin 3 μ g., thiamine hydrochloride 50 μ g., riboflavin 25 μ g., pyridoxine

hydrochloride 20 μ g., calcium pantothenate 100 μ g., nicotinic acid 100 μ g., folic acid 5 μ g., 2-methyl-1:4-naphthoquinone 10 μ g., inositol 5 mg., *p*-aminobenzoic acid 1 mg. The cod-liver oil concentrate supplied at least 300 i.u. vitamin A and at least 75 i.u. vitamin D/10 g. of diet. In the high-fat diet the content of fat was increased by adding an additional 20% of beef dripping and 10% of corn oil at the expense of sucrose to make the total content of fat 50%.

Effect of cold environment on acute choline deficiency

(1) Twenty male rats weighing from 170 to 220 g. were divided into two groups and were fed the basal hypolipotropic diet *ad lib.* for 14 days. During the experiment, one group was placed in a cold room maintained at a temperature of $1.5 \pm 1^\circ$, while the other was kept in the usual animal quarters at a temperature of $22 \pm 2^\circ$.

(2) Ten adult female rats (170–216 g.), which had been kept in the cold room for 54 days, were fed the hypolipotropic diet instead of their usual ration for a period of 2 weeks. At the same time ten female rats of similar weight range which had lived at 'room temperature' in the animal colony were exposed to cold and were fed the same hypolipotropic diet for a period of 2 weeks.

(3) The thyroid glands of ten adult female rats were removed surgically and the animals were given subcutaneous injections of 5 μ g. DL-thyroxine (Roche-Organon) daily for 5 days before and during exposure to cold. While in the cold room the animals were fed the basal hypolipotropic diet *ad lib.* The purpose of this procedure was to obviate the increased production of thyroid hormone which may occur after exposure to cold, and also to make possible the survival

of such animals in the cold environment (Sellers & You, 1950). Ten normal female rats were also kept in the cold room and received daily the average amount of basal diet consumed by the thyroidectomized rats. In both groups body weights ranged from 180 to 200 g. at the commencement of cold exposure.

(4) A hypolipotropic diet of high (50%) fat content was given *ad lib.* to two groups of ten female rats for 14 days. One group was maintained at room temperature, and the other was exposed to cold. A third group of ten females was exposed to cold simultaneously but was fed the basal (moderate fat) diet. The average body weight of all rats was 181 g. when the feeding was started.

The first part of this experiment was repeated using thirty female rats of approximately the same weight range. All rats received the same high-fat, low-choline diet *ad lib.*, but eighteen animals were kept in the cold room during the experimental period, while the remaining twelve lived at normal room temperature.

(5) A group of twenty female rats with body weights ranging from 162 to 190 g. were placed in the cold room and were fed the hypolipotropic diet *ad lib.* At the end of each week two animals were sacrificed, the last four rats being killed at the end of 10 weeks' exposure. The liver of each animal was removed and examined histologically. Other animals fed the same diet were sacrificed after a 15-week period of exposure to cold.

RESULTS

The excessive deposition of fat found in the livers of rats fed the hypolipotropic diet at room temperature did not occur in rats which were given the same diet but were kept in the cold environment. This preventive effect was also observed in rats which had previously been acclimatized to the cold environment. Similar results were obtained with thyroidectomized rats which received a constant daily maintenance dosage of thyroxine. The results are summarized in Table 1. Even after 10 and 15 weeks of exposure the fat content of the livers of rats fed the hypolipotropic diet remained low, in some cases within the normal range (Pl. 3).

When the content of fat in the basal diet was increased to 50%, the total lipid content of the livers was found to be higher than that of the group receiving the basal hypolipotropic diet in the cold environment. However, it was lower than the average figure obtained in the groups of rats which were fed the high-fat diet and were kept at room temperature (Table 2, A and B). The food intake of all rats exposed to cold was considerably higher than

Table 1. *Effect of cold environment (2°) on acute choline deficiency (14 days)*

No. of rats	Sex	Group	Environment	Average body wt. (g.)		Average food intake per day (g.)	Average liver wt. (g.)	Average total lipids (% wet liver wt.) ± S.D.
				Initial	Final			
10	M.	Normal	Cold	195	214	22	12.4	7.2±1.2
10	M.	Normal	Normal	196	248	15	13.8	24.8±4.9
10	F.	Acclimatized*	Cold	191	195	19	8.1	7.20†
8	F.	Normal	Cold	185	184	17	9.1	7.50‡
6	F.	Thyroidectomized†	Cold	189	182	16	8.3	6.75‡
6	F.	Normal	Cold	186	180	16	9.7	6.70‡

* Rats were in cold environment for 54 days before commencement of low choline diet.

† Each rat received 5γ thyroxine per day.

‡ Livers were pooled for fat determination.

Table 2. *Effect of cold environment on fat deposition in the liver with high-fat, choline-deficient diet (14 days)*

No. of rats (female)	Diet	Environment	Average body wt. (g.)		Average food intake per day (g.)	Average liver wt. (g.)	Average total lipids (% wet liver wt.) ± S.D.
			Initial	Final			
9	Basal	Cold	183	181	16.3	10.7	8.6*
9	High fat†	Cold	179	180	11.5	10.2	19.9± 9.7
8	High fat†	Normal	181	200	8.9	13.0	23.8±12.2 (P>0.10)
B							
14	High fat†	Cold	168	170	11.76	10.4	18.1± 8.1
12	High fat†	Normal	177	205	10.9	12.2	28.5±12.0 (P<0.02)

* Livers were pooled for fat determination.

† 50% fat in basal diet.

that of controls, but the rate of growth as measured by increase in body weight was lower than that of the control rats living at room temperature. The increased consumption of food was more marked in the diets with lower content of fat, and therefore with lesser caloric value.

DISCUSSION

When rats are fed diets low in choline and its precursors, the amount of excess lipid deposited in the liver may be influenced by a variety of factors. Some of these (e.g. intake of food, growth rate, age, sex, previous nutritional status, the presence or absence of other dietary constituents) have been appreciated for many years, others (e.g. environmental temperature, hormonal action) have received less attention. In the experiments reported in this paper the effect of lowering the environmental temperature has been studied, and it is apparent that a definite 'pseudolipotropic effect' due to cold took place. This effect was quite obvious when a diet of moderate fat content (20 %) was fed, but was less so with a diet of high (50 %) fat content. It is of interest to consider whether these results may be explained on the basis of one of the mechanisms described previously or whether some new explanation is necessary.

The energy requirement of a rat exposed to a cold temperature rises greatly, and in our experience the caloric intake is always increased. The rate of growth is adversely affected, but some increase in body weight still takes place. Rats of the range of weight studied have been observed in an environment of 1.5° for as long as a year and a half. In such circumstances it would be unreasonable to claim that the animals were failing to meet their energy requirements from dietary sources. At normal room temperature ($22 \pm 2^{\circ}$) the basal hypolipotropic diet described here must be restricted greatly in order to achieve the same lowering of liver lipids, and a failure to gain, or a fall in body weight occurs before excessive deposition of fat in the liver is decreased significantly (authors' unpublished data). From this it is concluded that a decrease in the ratio of caloric intake to energy and growth requirements (inanition) cannot alone explain the result.

The preventive action of the cold environment on deposition of fat in the liver is one of degree, however, for when the high-fat diet was fed, large amounts of lipid material were laid down. The average amount of high-fat (50 %) diet consumed was considerably less than that of the basal (20 % fat) diet. Because of the greater caloric values of the high-fat diet, this would be expected.

In studying the endocrine system, exposure to a cold temperature has been used frequently to elicit hormonal responses to conditions of 'stress'. Some of the reactions occur rapidly and are associated

with chemical and histological changes in the adrenal cortex. If the pseudolipotropic action of cold were concerned with this type of response, one might expect that the action would be transient in nature. After rats had been fed the basal hypolipotropic diet in the cold for 10 and 15 weeks, the fat content of the liver remained low. In 1950, Sellers, You, Ridout & Best observed that small daily doses (1-2 mg.) of cortisone given to normal rats fed a hypolipotropic diet failed to prevent the deposition of fat in the liver. Within a limited dosage schedule, this finding also applies to cortisone given orally, to deoxycorticosterone acetate and to adrenocorticotrophic hormone (ACTH) given by injection (Sellers, You, Ridout & Best, 1951). Thus it appears unlikely that adrenal stimulation is a principal cause of the effect.

A high dose of thyroid substance (0.8 % in the food) is necessary in order to produce a level of lipid approaching the normal, when this basal hypolipotropic diet is fed to rats. Even with such a high dosage, large, centrilobular extracellular fatty cysts are formed (Sellers & You, 1951), a feature seldom if ever seen in the livers of the animals exposed to cold. Therefore, the results obtained do not support the hypothesis that the thyroid plays an important part in the pseudolipotropic effect observed.

Acclimatization does not appear to alter appreciably the pseudolipotropic effect of cold. Acclimatized animals consumed considerably more food than did controls kept at room temperature, yet on chemical examination had significantly lower levels of fat in the livers.

No adequate explanation of the phenomenon can be advanced at present, yet an analysis of energy balance factors affords a basis for future work. The animal in the cold consumes a greater quantity of food than does the control kept at room temperature but does not grow so rapidly. In order to maintain body temperature in the cold, the energy expenditure is much greater and therefore it is logical to suggest that the greater caloric intake is used in producing heat. Very little dietary choline is available, so the necessary metabolic processes must be carried out (1) without choline, (2) by synthesis of choline within the body, or (3) the limited amount of choline present must be used more efficiently. Whichever of these alternatives is correct, it would appear that exposure to cold has brought about an alteration in normal metabolic pathways, and efforts are being made to investigate the possibilities more fully.

SUMMARY

1. When a hypolipotropic diet of moderate fat content (20 %) is fed to rats exposed to a temperature of $1.5 \pm 1^{\circ}$, excessive deposition of fat in the liver is effectively prevented.

2. This 'pseudolipotropic' action is a matter of degree, however, for with a high-fat (50 %), low-choline diet lipids accumulate in the liver. The fat content of the liver is significantly less than in control animals kept at room temperature on the same type of diet.

3. The pseudolipotropic action of cold is demonstrable in acclimatized as well as in normal animals, and is apparently not mediated to a recognizable

extent through either the thyroid or the adrenal gland.

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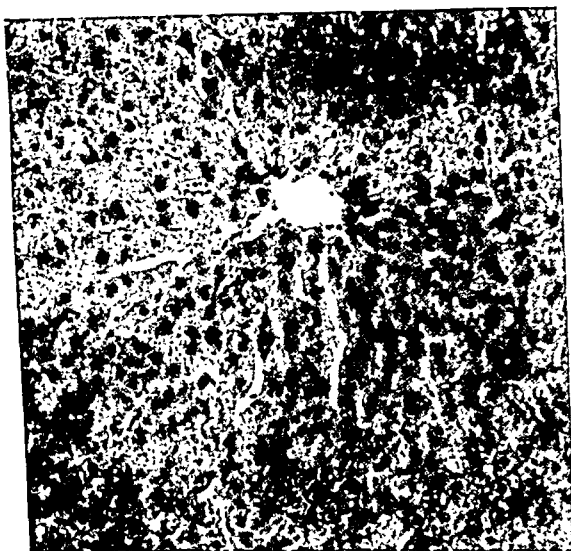
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EXPLANATION OF PLATE 3

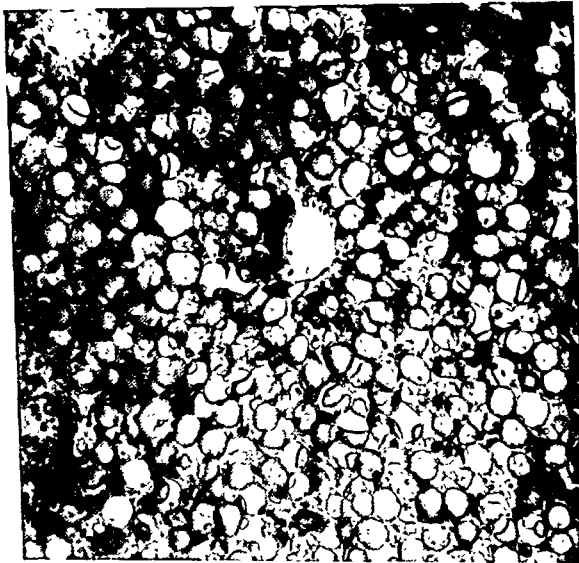
- (a) Livers from (left) a rat fed the hypolipotropic diet for 8 weeks in an environment of 1.5°, and (right) from a rat fed the same diet for the same period at room temperature. The liver from the control animal is yellowish in colour and is much larger than that of the rat kept in the cold.
- (b) Photomicrograph of liver tissue (Orange red O × 135) from a rat fed a hypolipotropic diet for 8 weeks in an environment of 1.5°. The appearance of the section is essentially normal.
- (c) Photomicrograph of liver from rat fed the same diet but kept at room temperature. Note the globules of fat deposited throughout the lobule.



(a)



(b)



(c)



The Immunological Properties of Proteins Treated with Di-2-chloroethylmethylamine

By WINIFRED M. WATKINS* AND A. WORMALL

Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1

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When proteins are treated with 'sulphur mustard' (mustard gas; di-2-chloroethyl sulphide) they undergo a slight modification of their immunological properties, and the injection of mustard gas-treated proteins into rabbits may lead to the formation of antibodies capable of reacting specifically with any mustard gas-treated protein (Berenblum & Wormall, 1939; Bourns, Francis & Wormall, 1946*a*). It was suggested (Berenblum & Wormall, 1939) that acquired hypersensitivity to mustard gas might be partly due to an immunological change in the tissue proteins exposed to mustard gas, i.e. to the formation of 'foreign' proteins and subsequent production of specific antibodies. This view was later supported by the induction of hypersensitivity in laboratory animals by the application of mustard gas (Holiday, 1942; Kidd & Landsteiner, 1944; Moore, 1944). More recently, Fleming, Moore & Butler (1949) have shown that in the presence of phosphate buffer at pH 7.5 or 9, mustard gas reacts with proteins to give complexes which contain residues of diethyl sulphide, phosphate and protein, and that when the mustard gas-protein complexes prepared with added phosphate at pH 8-9 are injected into rabbits they give rise to strongly precipitating antisera.

The nitrogen mustards also react with a few enzymes and other proteins. Di-2-chloroethylmethylamine ($\text{CH}_3\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), e.g., strongly inhibits hexokinase (Dixon & Needham, 1946), cholinesterase (Thompson, 1947; Adams & Thompson, 1948), and choline oxidase and choline acetylase (Barron, Bartlett & Miller, 1948). It has been shown that this nitrogen mustard reacts with the amino, carboxyl, sulphydryl, sulphide and pyridine groups of proteins (cf. Fruton, Stein & Bergmann, 1946; for other references see Gilman & Philips, 1946; Boyland, 1948; Philips, 1950; Karnofsky, 1950) and that it rapidly inactivates the protein components of haemolytic complement (Watkins & Wormall, 1948, 1952).

In view of the fairly widespread clinical use of the nitrogen mustards for the treatment of Hodgkin's and certain other neoplastic diseases, we decided to

study the immunological properties of proteins treated with the nitrogen mustards. One of the objects of this work was to determine whether antibodies specific for nitrogen mustard-treated proteins could be produced by the repeated injection of nitrogen mustard or nitrogen mustard proteins; any antibody response of this type might indicate the possibility of hypersensitivity occurring in patients subjected to prolonged treatment with the nitrogen mustards. It was also hoped that these immunological and chemical studies might yield information about the mode of action of the nitrogen mustards on amino-acids, peptides and proteins.

EXPERIMENTAL

Nitrogen mustard (NM). The only NM used in these experiments was di-2-chloroethylmethylamine hydrochloride (code no. HN2). After the addition of the solid to protein solutions, the mixtures were shaken gently until the solid had dissolved. When NM was intravenously injected into rabbits, the solid was quickly dissolved in a small volume of 0.9% (w/v) NaCl and the solution injected immediately.

Ovalbumin. Hen ovalbumin, prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation, was recrystallized three times (cf. Cole, 1933).

Antigens

The general method used for the preparation of the NM-treated proteins (NM proteins) was as follows. The NM was added, in portions, to the protein solution kept at 37°, and 5% (w/v) Na_2CO_3 was added to the mixture, when required, to maintain the pH at about 7.5, or in a few experiments 7.5-8.0. One-quarter of the NM was added at the start and the same amount about 4 hr. later, and this treatment was repeated the next day. On the third day the mixture was again kept for 8 hr. at 37°, this time without further addition of NM. The mixtures were kept overnight in the refrigerator during this period.

With a few exceptions (the antigens designated A, C and E below) the products were then dialysed against frequently changed 0.9% NaCl for 48 hr. at 4°, to remove transformation or 'hydrolysis' products of NM. Dilute HCl or Na_2CO_3 was added where necessary to bring to pH 7.4, Merthiolate (0.01%) was added as a preservative, and the solutions were stored at 0-4°. Early in these investigations we observed that the NM used has a very powerful bactericidal action, but we continued to add Merthiolate to the antigen solutions

* Present address: Lister Institute of Preventive Medicine, London, S.W. 1.

prepared for storage since it was considered possible that the bactericidal activity of the nitrogen mustard might be due to some of its unstable cyclic transformation products.

When most of this work had been completed, the paper by Fleming *et al.* (1949) appeared, describing the effect of phosphate on the reaction between ordinary mustard gas and proteins. Since it seemed possible that phosphate might similarly affect the reaction between NM and proteins, a few further experiments were made with antigens prepared in the presence of extra phosphate.

The following antigens were prepared by the general method described above, with occasional slight modification (detailed below). These antigen preparations contained hydrolysis products of NM. No attempt was made in the preparation of antigens *A*, *C* and *E* to remove these impurities since it was thought that the treatment involved, for example, dialysis or the precipitation of the protein complexes by ethanol, might split NM-protein linkages. Control tests showed that any hydrolysis products of nitrogen mustard likely to be present after these antigen preparations had been kept for several weeks would not in any way affect precipitin or complement-fixation tests.

Antigens for immunization

(i) *NM ovalbumin*. Preparation *A* was made by the action of NM (1.5 g.) on a solution of crystalline ovalbumin (100 ml. containing 7.6 g. of protein). Preparation *B* (extra phosphate) was made similarly, but 0.5M-phosphate buffer of pH 8.0 (50 ml.) was added to the ovalbumin solution before addition of the NM.

(ii) *NM horse serum proteins*. Preparation *C*: normal horse serum (100 ml.) was treated with NM (0.80 g.). Preparation *D* was made similarly, with 0.5M-phosphate buffer of pH 8.0 (50 ml.) added to the horse serum.

Test antigens

In the precipitin and complement-fixation tests, the following antigens were used in addition to the above-described immunizing antigens. All the test-antigen solutions had a pH of about 7.4.

(i) *NM rabbit serum proteins*. Preparation *E*: normal rabbit serum (10 ml.) was treated with NM (80 mg.). The same method was used for preparations *F* (extra bicarbonate) and *G* (extra phosphate), except that additional buffers, 0.5M-NaHCO₃-H₂CO₃ (pH 7.5) (5 ml.) and 0.5M-phosphate (pH 8.0) respectively, were added to the rabbit serum.

(ii) *NM gelatin*. Preparation *H*: NM (300 mg. added in three 100 mg. portions at intervals of a few hours) was added to a mixture of a gelatin solution (500 mg. in 30 ml.) and 0.5M-NaHCO₃ (50 ml.) and the solution kept at 37° and pH 7.5-8.0 for 48 hr. In the preparation of the 'phosphate-antigen' (*J*) 0.5M-phosphate buffer (20 ml.) was used instead of NaHCO₃.

NM-treated amino-acids. Many attempts were made to prepare NM-amino-acid derivatives for use in precipitin-inhibition tests. Details of the methods used are given in the text.

Methods

Nitrogen determinations. Total N was determined by the micro-Kjeldahl method, using the Markham (1942) distillation apparatus. The Van Slyke (1912) volumetric method was used for the determination of α -amino N.

Immunization. In view of the weak specific antibody response following the injection of proteins treated with sulphur mustards (Berenblum & Wormald, 1939; Bournsnel *et al.* 1946a), we decided to use several immunization methods in our attempts to get antibodies specific for NM proteins.

(a) *Intravenous injection of NM*. Solutions of NM hydrochloride in 0.9% NaCl were injected into the ear veins of rabbits on 4 successive days. After an interval of 12 days each rabbit received a further course of four similar injections, except no. 572 which received two injections only, because of badly damaged ear veins. The amounts of NM hydrochloride injected at each injection were 0.1 mg. (rabbits nos. 523 and 563), 0.2 mg. (nos. 614 and 619) and 0.3 mg. (nos. 572 and 576) per kg. of body weight. Blood samples were taken 7 and 14 days after the final injections, and the sera were tested for antibodies capable of reacting with NM proteins.

(b) *Intramuscular injection of alum-precipitated antigens*. The protein complexes were precipitated by potassium alum at pH about 6.5, as recommended by Proom (1943), and each rabbit received an intramuscular injection of 5 ml. of a suspension (containing about 17 mg. of protein/ml.) into each hind leg. A course of three injections was given, at intervals of 12-14 days, and the sera were tested 10 days after each injection.

(c) *Intraperitoneal injections*. The antigen solution (5 ml.) was injected intraperitoneally, and each rabbit, except no. 686 which died 10 days after the third injection, received five injections at intervals of 9-12 days. Precipitin tests were made on the blood serum samples taken 9 or 10 days after the third and subsequent injections.

In all, twenty-seven rabbits were immunized by methods (b) and (c), and details of the antigens and the mode of immunization are given in Table 1. The antisera were filtered through Berkefeld filters and stored in sterile tubes at 0-4°.

Table 1. *Details of immunizations with NM proteins*

(IM, intramuscular injections of the alum-precipitated antigen; IP, intraperitoneal injections.)

Group	Rabbits (nos.)	Immunizing antigen	Mode of injection
1	731, 744, 746, 747	NM horse serum (preparation C)	IM
2	734, 750, 751, 754	NM ovalbumin (preparation A)	IM
3	556, 654, 671, 708, 727	NM ovalbumin (preparation A)	IM
4	795, 809, 841	NM ovalbumin* (preparation B)	IP
5	679, 681, 686	NM horse serum* (preparation D)	IP
6	689, 719, 837, 840	NM ovalbumin* (preparation B)	IM
7	666, 692, 797, 854	NM horse serum* (preparation D)	IM

* Antigens prepared in the presence of extra phosphate.

Precipitin tests. These were made as described previously (Hopkins & Wormald, 1933). The tests with NM gelatin as test antigen were made at room temperature.

Precipitin-inhibition tests. The substance or mixture being tested for inhibitory power was dissolved in 0.9% NaCl to give a 1% (w/v) solution, or, with sparingly soluble substances, a saturated solution (at room temperature) was prepared. The solution was neutralized and varying amounts (1-4 vol.) were added to the antigen solution (1 vol.); 0.9% NaCl was then added (to give a total of 5 vol.). Antiserum (2 vol.) was added and the mixtures were shaken and kept at 37°. The extent of precipitation was noted after 15 min., 1 and 3 hr.

Complement-fixation tests. These were carried out as described previously (Berenblum & Wormall, 1939), but with a sensitized sheep erythrocyte system.

RESULTS

Intravenous injection of NM into rabbits

The sera of six rabbits which were given frequent injections of NM contained no antibodies capable of precipitating NM ovalbumin. Thus no evidence was obtained that the injection of NM leads to the production of antibodies specific for NM proteins, even when the amount injected is appreciably greater than that injected into patients suffering from Hodgkin's and some other neoplastic diseases. The amount of NM injected into man varies very considerably, but a widely adopted method is a course of injections of 0.1 mg./kg. of body weight on each of 4 successive days, with a second similar course some weeks or months later. In our experiments doses three times as large as these, with a second course after an interval of 12 days, failed to elicit the formation of antibodies specific for NM proteins.

Injection of NM proteins

Although antibodies specific for NM proteins could not be detected in the serum of rabbits following the injection of NM, the possibility of antibody production in the tissues of these injected animals cannot be excluded. We decided, therefore, to study the effect of injecting preformed NM proteins. Horse serum proteins and ovalbumin, previously treated with NM, were injected intraperitoneally or intramuscularly (as the alum-precipitated antigen) into several groups of rabbits, and the sera of these animals were tested at intervals for antibodies capable of reacting, in precipitin and complement-fixation tests, with various NM-proteins.

Some of the injected rabbits had no detectable 'NM protein-specific' antibodies in their serum at any time during the long immunization; all produced antibodies which precipitated the injected antigen or the corresponding untreated protein (horse serum proteins or ovalbumin), showing that the injected complexes were fully antigenic. Thus treatment with considerable amounts of NM at pH 7.5-8 does not destroy the antigenicity of ovalbumin or the serum proteins.

The sera of seven of the twenty-seven injected rabbits gave, at some stage in their immunization, weak but significant precipitin reactions with NM proteins different from those used for immunization. For example, the injection of NM horse serum proteins occasionally gave rise to antibodies which

Table 2. *Precipitin reactions with antisera to proteins treated with nitrogen mustard (NM)*

(Precipitin reactions are recorded, as follows: -, (no reaction); tr., (trace); ±, +, +±, ++ etc., in increasing degrees of precipitation.)

Antigen	Concentration of antigen solution (%)	Antisera against			
		NM ovalbumin		NM horse serum proteins	
		No. 751	No. 754	No. 746	No. 747
NM ovalbumin	0.25	+++	+++	-	-
	0.05	+++	+++	-	-
	0.01	++	++	±	-
	0.002	-	tr.	-	-
NM horse serum proteins	0.25	-	-	+±	+±
	0.05	±	-	+ +±	+ +±
	0.01	±	±	+	+
	0.002	-	-	tr.	-
NM rabbit serum proteins	0.25	-	-	-	-
	0.05	tr.	tr.	-	-
	0.01	-	-	±	±
	0.002	-	-	tr.	tr.
Ovalbumin	0.25	+±	+±	-	-
	0.05	±	±	-	-
	0.01	tr.	tr.	-	-
	0.002	tr.	tr.	-	-
Horse serum proteins	0.25	-	-	-	-
	0.05	tr.	tr.	+±	+±
	0.01	-	-	+ +±	+ +±
	0.002	-	-	+±	+±

gave precipitates with NM ovalbumin or NM rabbit serum proteins, but not with NM gelatin. Typical results of these reactions are given in Table 2. No evidence was obtained that the specific antibody response depended on the particular NM protein injected or on the mode of immunization. The results with the NM protein antigens prepared in the presence of phosphate were not significantly different from those obtained with antigens prepared without added phosphate.

Complement-fixation tests fully confirmed this observation that antibodies specific for NM proteins were occasionally produced in the injected rabbits. Typical results (Table 3) showed that the sera of some of the rabbits injected with NM ovalbumin reacted with heterologous NM proteins, e.g. NM horse serum proteins. No complement fixation was obtained when normal horse serum proteins were used as the antigen in these tests with antisera to NM ovalbumin.

Table 3. *Complement-fixation tests with antisera to NM ovalbumin*

(Mixtures of 0.5 ml. of the diluted antigen (of concentration recorded in column 1), 0.10 ml. of antiserum (previously heated at 56° for 20 min. to inactivate any complement present) and 0.10 ml. of dilute guinea pig serum (1 vol. of serum plus 4 vol. of 0.9% NaCl) were kept at room temperature for 1 hr. After the addition of 0.5 ml. of a 3% suspension of sensitized sheep erythrocytes to each tube, the mixtures were shaken and kept at 37°, and the extent of haemolysis was noted at intervals. The readings recorded in this table were those after 1 hr. Degrees of haemolysis; 0, none; tr., trace; 1, 2 and 3, increasing degrees of haemolysis; 4, complete haemolysis.)

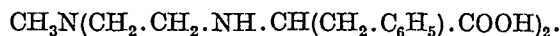
Concentration of antigen (NM horse serum proteins) (mg./100 ml.)	Degree of haemolysis	
	Antiserum no. 750	Antiserum no. 734
250	4	4
83	4	4
28	3	4
9.3	2	3
3.1	tr.	2
1.0	0	1
0.34	0	0
0.11	0	0
0.038	0	0
0.013	0	tr.
0.004	tr.	1
None (control)	4	4

Attempts to obtain specific inhibition of the reaction between NM proteins and their antibodies

Our immunological and chemical evidence suggests that the slight change in the immunological properties of proteins effected by NM is due to the combination of NM and the protein. It is conceivable, therefore, that a suitable NM amino-acid or NM peptide might be found which would

specifically inhibit the NM protein antigen-antibody reaction, for example the precipitin reaction between NM ovalbumin and antibodies to NM horse serum globulins. Specific inhibition of this type would, of course, provide useful information about the nature of the protein groups to which the NM becomes attached.

Attempts were made to isolate products formed by the action of NM on various amino-acids, and in these experiments, as in the preparation of NM protein antigens, we kept the conditions as near as possible to the physiological, namely temperatures not exceeding about 37° and a pH of 7.5–8.5. Fruton, Stein & Bergmann (1946) describe the reduction of free amino nitrogen which occurs when solutions of various amino-acids are shaken with NM at pH 8–8.5 and 25°, and they isolated from NM plus phenylalanine a reaction product to which they gave the formula



Our efforts to prepare this pure substance have, unfortunately, been unsuccessful, and so have our attempts to obtain pure NM derivatives of glycine, lysine and cysteine. The method we used was essentially that described by Fruton, Stein & Bergmann (1946), and although with all these amino-acids we observed a considerable reduction (24–41%) in the free amino nitrogen and a complete loss of free SH when cysteine was used, we were unable to separate crystalline reaction products; in some instances we attempted, without success, to separate the NM amino-acid derivatives as copper salts, a method found satisfactory for a mustard gas derivative of glycine (Boursnell, Francis & Wormall, 1946b). Since, however, the relatively crude products differed from the original amino-acids in total nitrogen and free amino-nitrogen contents, it was decided to find out whether they specifically inhibited the NM protein antigen-antibody reaction.

In a further search for inhibitory substances and as part of a more general study of the action of NM on proteins, we investigated the action of NM on nucleic acid and glutathione.

(a) Yeast nucleic acid (British Drug Houses) was purified by dissolving the sodium salt in water and precipitating the acid at pH 1.5–3.0. After it had been washed successively with ethanol and ether and dried at 50°, the product (0.5 g.) was dissolved in 0.5M-NaHCO₃-H₂CO₃ buffer of pH 7.5 (20 ml.) and mixed with a freshly prepared neutralized solution of NM (0.25 g.) in about 4 ml. of water. The mixture was kept at room temperature for 24 hr. and three fractions were separated by treatment with N-HCl: (A) a light-brown solid (0.43 g.) precipitated over the range pH 2–4, (B) an almost white solid (0.06 g.) precipitated at pH 1–2, and (C) a white solid (0.03 g.) precipitated from the mixture at pH 1, after the removal of fraction (B), by adding excess of ethanol. All these fractions were washed with ethanol and then with ether, and finally dried *in vacuo*. They

were not sufficiently pure for complete analysis, but N and P determinations were made on the two largest fractions (A) and (B); these had N/P ratios of 2.06 and 1.95 respectively, compared with a ratio of 1.79 for the untreated purified nucleic acid.

(b) A freshly prepared solution of NM (400 mg.) in 0.5M- $\text{NaHCO}_3\text{-H}_2\text{CO}_3$ buffer of pH 7.5 (5 ml.) was added to a solution of glutathione (300 mg.) in NaHCO_3 buffer (5 ml.) and the mixture was kept at room temperature. After 30 min. there was no free SH detectable by nitroprusside, and in the course of 48 hr. there was a 48% reduction in the free amino nitrogen of the solution. The mixture was then concentrated to a syrup *in vacuo* over H_2SO_4 at room temperature, and the crystalline material which separated was rubbed with acetone to give a flocculent powder which was washed with acetone and dried. The remaining syrup was kept for some time *in vacuo* over conc. H_2SO_4 , and the gelatinous solid obtained was rubbed with acetone, giving a powder which was washed with acetone and dried. In view of the inability of these products to inhibit the NM protein serological systems, no attempts have so far been made to purify and identify them.

Precipitin inhibition tests with the above-mentioned NM-treated amino-acids, nucleic acid and glutathione showed that these products had no specific inhibitory effect on the NM protein antigen-antibody system. Where inhibition occurred it was of a non-specific nature, for the product concerned inhibited unrelated precipitin reactions. No inhibition was obtained with some 'hydrolysis' products of NM (namely di-2-hydroxyethylmethylamine, 2-chloroethyl-2-hydroxyethylmethylamine and the dimer, NN' -di(2-chloroethyl)- NN' -dimethylpiperazinium chloride), or with the following NM derivatives; a crystalline compound formed by the interaction of NM and di-2-hydroxyethylmethylamine (Fruton, Stein, Stahmann & Golumbic, 1946) and the hexamethylenetetraminium derivative formed by the action of NM on hexamethylenetetramine (Gurin, Deluva & Crandall, 1947).

The stability of the NM protein complexes

It was not possible in these investigations to determine the number of NM residues present in the NM protein complexes, and indeed this could only be achieved in later investigations with ^{15}N -labelled NM (Burnop, Richards, Watkins & Wormald 1951). A few preliminary qualitative experiments were made, however, to determine the stability of the linkages between the NM and proteins, and whether the treatment of serum proteins with NM produces protein derivatives which are precipitated, as are the corresponding mustard gas sulphone proteins, by the addition of dilute acid.

Dialysis of NM horse serum proteins for 4 days at 4° against frequently changed 0.9% NaCl, or their maintenance in acid or alkaline solution (over the range pH 3-9) for 16 hr. at 37° and subsequent

dialysis against 0.9% NaCl at 4°, effected no significant loss of the power of these NM proteins to give precipitates with antisera to NM ovalbumin. A slight loss of precipitability occurred in solutions at pH 11 kept for 16 hr., but this reduction was most probably due to denaturation rather than removal of NM groups, since there was a corresponding decrease in the capacity of the NM protein complex to precipitate antibodies to untreated horse serum globulins.

With regard to precipitation by acid, it was found that the NM serum proteins, prepared by the action at pH 7.5 of either freshly prepared aqueous solutions of NM or NM solutions which had been allowed to hydrolyse (for example, at 37° for 4 hr. followed by 18 hr. at 4°), could not be precipitated by adding dilute acid. In this respect, therefore, the action of NM resembles that of 'sulphur mustard' and differs from that of mustard gas sulphone.

DISCUSSION

The extensive work of Landsteiner and his colleagues (for the literature, see Landsteiner, 1936, 1945) has shown that many simple organic compounds produce, when introduced into the animal body, a condition of hypersensitivity because they react with the body proteins to form immunologically foreign conjugates. Induced hypersensitivity to ordinary mustard gas has been explained in this way (Berenblum & Wormald, 1939), and if NM combines firmly with proteins the risk of producing allergic reactions by injecting nitrogen mustard must be considered.

Our investigations show that when the serum proteins and ovalbumin are treated with nitrogen mustard under physiological conditions of pH and temperature, these proteins undergo modifications in their immunological properties. The complexes formed produce antibodies specific for NM-treated proteins in some, but not all, of the injected rabbits. This alteration in the immunological properties of the proteins is, however, very slight, since specific antibody formation was only occasionally produced. NM undoubtedly combines firmly with proteins, as was indicated by the investigations described here and more fully established by our quantitative studies with nitrogen mustard labelled with ^{15}N (Burnop *et al.* 1951), and it is surprising that the specific antibody production following the injection of NM-treated protein is not stronger and more frequent. The specific antisera produced by the injection of sulphur mustard-proteins were not very potent (Berenblum & Wormald, 1939; Bournsnel *et al.* 1946a), but they were more powerful than the antisera to NM proteins produced in the experiments described here, and they were obtained in a higher percentage of injected animals.

This infrequency of specific antibody production following the injection of NM proteins may be due to the fact that relatively few NM residues become attached to each protein molecule, and since some of these groups can be detached by long dialysis (Burnop *et al.* 1951), the injected NM proteins may soon lose many of their determinant NM groups. Another possible explanation is that NM might act on proteins mainly by a cross-linking action. Haddow, Kon & Ross (1948) showed that the cytotoxic activity of a series of halogenoalkylarylamines could be related to the presence of two halogenoalkyl groups in the molecule, and Goldacre, Loveless & Ross (1949) suggested that cross-linking might account for the action of the nitrogen mustards in producing chromosome abnormalities (see also Loveless & Revell, 1949; Loveless, 1951). According to this view two reactive groups are required in the NM molecule to permit it to react at two distant points either on the same fibre or on two contiguous fibres. Cross-linking of this type might explain why NM-treated proteins differ so little immunologically from the unchanged protein, for with a cross-linking mechanism there would be no characteristic determinant grouping projecting from the NM protein molecule.

Whatever the explanation, it appears that the injection of NM or of NM-treated proteins into rabbits rarely leads to the production of antibodies specific for NM proteins and this is supported by clinical evidence. There are apparently few, if any, cases of allergic reactions in man attributable to the intravenous injection of the nitrogen mustards. Furthermore, Hartman, Mangun, Feely & Jackson (1949) found no evidence of antigenic effects when man and dogs were given repeated injections of plasma to which NM had been added as a preservative. On the other hand, Moore & Rockman (1950) have recently found that skin hypersensitivity to one of the nitrogen mustards (di-2-chloroethyl-ethylamine) can be induced in man by a single NM burn or preferably two successive burns with a week's interval between the applications of NM; no evidence was obtained of simple cross-reactions between sulphur mustards and NM in these sensitivity tests. These observations of Moore & Rockman suggest that the possibility of occasional production of hypersensitivity to NM should not be overlooked.

Nitrogen mustards such as di-2-chloroethyl-methylamine react with many groups in the protein molecule (cf. reviews by Philips, 1950, and Karnofsky, 1950) and they degrade deoxyribonucleic acid solutions as shown by a decrease in viscosity (Gjessing & Chanutin, 1946; Butler, Gilbert &

Smith, 1950; Butler & Smith, 1950). It is not known, however, which parts of the protein molecule are primarily concerned when NM effects its characteristic biological actions, including the slight serological change described in this paper. Our attempts to obtain information on this point by serological inhibition tests have so far been unsuccessful, for we have not been able to obtain any NM amino-acid derivative or any similar preparation which will specifically inhibit the reaction between NM proteins and their antibodies. With the aid of NM labelled with ^{15}N and ^{14}C we are making a further study of the action of NM on proteins and tissue constituents, and it has now been possible to determine the amount of NM which combines firmly under physiological conditions with the serum and other proteins (Burnop *et al.* 1951). Quantitative data about the combination of NM and nucleic acids has also been obtained (Burnop, Richards & Wormall, 1952), and it is hoped that these quantitative studies will throw some light on the immunological results reported here.

SUMMARY

1. Antibodies capable of reacting with NM (nitrogen mustard)-treated proteins could not be detected in the sera of rabbits which had received a series of intravenous injections of di-2-chloroethyl-methylamine, one of the nitrogen mustards frequently used therapeutically.

2. The sera of rabbits which had been given a course of injections of NM-treated proteins (horse serum proteins or ovalbumin treated with NM at 37° and pH 7.5–8.0) occasionally contained antibodies capable of reacting specifically with NM proteins, including NM-treated rabbit serum proteins.

3. This specific antibody production was, however, very weak and infrequent. It is concluded that the risk of hypersensitivity following frequent exposure to NM or a series of intravenous injections of the drug, is slight, at least with the rabbit.

4. The complexes formed when NM acts on serum proteins or ovalbumin are comparatively stable, and the NM protein does not lose its serological specificity as a result of long dialysis. Attempts to obtain a NM amino-acid compound capable of specifically inhibiting the NM protein antigen-antibody reaction have been unsuccessful.

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The Determination of Plasma Fibrinogen by the Clot-Weight Method

By G. I. C. INGRAM

Department of Surgery, University of Edinburgh

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The clot-weight technique is a convenient method for the determination of plasma fibrinogen. The result is obtained as fibrin with sufficient accuracy for clinical purposes. Nevertheless, certain possible causes of error appear not to have received attention and these have been investigated.

METHODS

Plasma. 9 vol. of human blood are received from a paraffined syringe into 1 vol. of sodium citrate solution containing 3.8 g./100 ml. and the mixture is centrifuged at 2000 rev./min. for 30 min.

Technique of fibrinogen determination. The citrated plasma is diluted with an approximately equal vol. of 0.025 M-CaCl₂ solution and allowed to stand at 37°. The fibrin is wound off

on to a wooden applicator until no further clot forms, washed in distilled water, rolled off the rod, drained between filter papers, pulled out thin (when of sufficient bulk), dehydrated first in acetone and then in air at 105° for 3 hr., and weighed.

From the haematocrit of the citrated blood the calculation is made as follows:

$$\text{Plasma fibrinogen content} = \frac{\text{clot weight}}{\text{sample volume}} \times \frac{\text{volume of citrated plasma in 10 ml. citrated blood sample (from haematocrit)}}{\text{volume of citrated plasma} - \text{volume of citrate}}$$

This correction ignores the variable effect of the citrate upon the cell-plasma fluid partition, but error from this cause is thought to be small compared with differences between persons.

EXPERIMENTAL

Formal experiments were made to determine the effect of certain variables in technique which in practice it may not always be possible to standardize.

Effect of sample storage. It may not always be possible to make determinations on fresh samples. Beginning, therefore, 3 hr. after the blood was drawn, triplicate determinations were made on 8 successive days (except the fifth) on 5.0 ml. samples from a quantity of plasma stored in glass at about 4°.

The data are shown in Table 1. Analysis of the results showed a significant linear regression increasing on days over the 8 days, but as no effect was apparent until the fourth day after venepuncture, storage errors would appear to be seldom of practical consequence.

Table 1. *Effect of storage on clot weight*

(Weights of clots from 5.0 ml. portions of a sample of plasma stored at 4° and tested 3 hr. after withdrawal (day 0) and on subsequent days for 1 week.)

Day	Replicate clot weights (mg.)			
	1	2	3	Mean
0	14.0	14.0	14.5	14.17
1	14.1	14.0	13.8	13.97
2	14.5	—	14.3	14.37
3	14.3	13.9	14.4	14.20
4	14.7	14.5	14.4	14.53
6	14.7	14.5	14.4	14.53
7	14.4	14.2	14.6	14.40

Effect of sample volume. From one sample of plasma, three replicate determinations were made on subsamples of 5.0, 4.0, 3.0, 2.0 and 1.0 ml. In eight clinical determinations replicates had also been made on unequal volumes.

The data from the formal experiment are shown in Table 2. Comparative analyses were made on the two groups of results. The analysis of the fifteen experimental determinations showed a significant

Table 2. *Effect of sample volume on clot weight*

Sample vol. (ml.)	Replicate fibrin determinations (mg. fibrin/ml. plasma)			
	1	2	3	Mean
5.0	2.08	2.18	2.16	2.14
4.0	2.18	2.15	2.25	2.19
3.0	2.20	2.20	2.20	2.20
2.0	2.25	2.25	2.25	2.25
1.0	2.20	2.20	2.30	2.23

negative linear regression on volume, equivalent to an addition of about 1.3 % to the clot weight for each ml. decrease in volume below 5.0 ml. A similar (but non-significant) association was observed in the eight field determinations, differing from subject to subject, but equivalent, on the average, to an addition of 3.6 % wt./ml. decrease in volume.

Comparison of recalcification with added thrombin for fibrin isolation. As it is theoretically possible that sufficient thrombin to clot its fibrinogen content might not be available from a given sample of plasma, it might be preferable to cause clotting by the addition of thrombin (Jones & Smith, 1930). Nineteen parallel determinations were therefore made on fifteen plasma samples (none from patients known to be suffering from one of the haemorrhagic diseases) by the recalcification technique given above and by diluting with 2 or 3 vol. of saline and then adding thrombin solution until no further clot formed.

Table 3. *Comparison of fibrinogen concentration determined with thrombin and by recalcification*

Plasma samples	Fibrinogen concentration (g./100 ml.)	
	Fibrin obtained by adding thrombin	Fibrin obtained by recalcification
1	0.37	0.37
2	0.74	0.67
3	0.44, 0.45, 0.42	0.46, 0.46, 0.41
4	0.63	0.60
5	0.38	0.36
6	0.37	0.41
7	0.57	0.49
8	0.61, 0.61	0.64, 0.59
9	0.36	0.32
10	0.48, 0.45	0.59, 0.48
11	0.48	0.39
12	0.43	0.46
13	0.29	0.39
14	0.48	0.55
15	0.59	0.59

The data are shown in Table 3. The range of values obtained on the fifteen samples was 0.29–0.74 g./100 ml. and inspection shows no systematic difference between the series at high or at low levels. The mean difference between the two series was 0.006 g./100 ml. in favour of thrombin; $t=0.042$ ($P>0.9$). The two methods are clearly equivalent on the basis of this experience.

Effect of dilution before recalcifying. It is usually recommended that the plasma be diluted with 10 or 20 vol. of saline before recalcifying, but this might lead to significant loss from the solubility of fibrin in physiological salt solutions. Six 5.0 ml. replicates were determined by the described procedure in parallel with six similar replicates from the same sample diluted to 45 ml. with 0.9 % (w/v) sodium chloride and then recalcified with 5.0 ml. 0.025M-calcium chloride.

The data are shown in Table 4. The mean clot weight of the six diluted replicates was 13.73 mg. and of the six undiluted replicates, 14.03 mg.; the difference in means was 0.30 ± 0.11 , which is significant ($0.02 < P < 0.05$). The lower, diluted value, a decrease of about 2 % on the undiluted value,

suggests that a little fibrin was lost by solution; the described procedure was therefore preferred: in this also, the manipulation of fibrin was found to be easier in the smaller volume.

Table 4. *Effect of dilution on clot weight*

(Weights of clots from twelve 5.0 ml. portions of one sample of plasma: in six instances the recalcified mixture was diluted five times.)

Replicates	Clot weight (mg.)	
	Diluted	Undiluted
1	14.1	14.1
2	13.9	14.0
3	13.6	13.8
4	13.6	14.1
5	13.8	14.1
6	13.4	14.1
Mean	13.73	14.03

Ash weight of clot. To exclude systematic error from mineral content (Foster & Whipple, 1922), two groups of three clots from 5.0 ml. normal plasma samples were pooled and weighed. Thereafter they were ashed and re-weighed. In neither instance did the combined weight of ash from the pooled clots exceed the limit of balance error (± 0.1 mg.).

DISCUSSION

Effect of sample volume. The inverse relation of fibrinogen concentration to the plasma sample volume is curious. It is interesting that a similar

inverse relation is apparent in the fibrin dry-weight data of Foster & Whipple (1922) and of Howe (1923), though in neither report does it receive comment; in Howe's data the magnitude of the effect is very small. The error, about 5% or less per ml. below 5 ml., is obviously small compared with differences expected between persons, if approximately the same volume of plasma, 3–4 ml., is always used.

Effect of sample dilution. The findings suggest that plasma samples should not be much diluted at recalcification, although again the error, a loss of 2% on clot weight with fivefold dilution of the reaction mixture, is small compared with differences to be expected between persons.

SUMMARY

1. Daily determinations on a stored plasma sample showed a significant increase of clot weight from the fourth day after withdrawal.

2. There is an inverse relation between sample size and determined fibrinogen concentration.

3. Clot weights obtained by recalcification agreed well with those obtained by adding thrombin.

4. Dilution of the sample at recalcification reduced the weight of clot obtained.

5. The ash weight of the clot was negligible.

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Bacterial Arylsulphatase

By J. E. M. WHITEHEAD

Department of Bacteriology, St Thomas's Hospital Medical School, London, S.E. 1

AND A. R. MORRISON AND L. YOUNG

Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

(Received 31 December 1951)

It is known that in plants and animals there occur enzymes which catalyse the hydrolysis of sulphuric acid esters. These enzymes, the sulphatases, have recently been reviewed by Fromageot (1950). The sulphatases differ with respect to the type of sulphuric acid ester upon which they act, and they include arylsulphatase (phenolsulphatase), which hydrolyses arylsulphuric acids. The existence of

arylsulphatase was first demonstrated by Derrien (1911) who observed that extracts of the shell fish, *Murex trunculus*, hydrolyse potassium indoxyl sulphate. An enzyme with similar activity was later found to be present in *Aspergillus oryzae* by Neuberg & Kurono (1923), and when evidence became available concerning its specificity it was named 'phenolsulphatase' (Neuberg & Simon, 1932).

Recently, the more satisfactory name 'arylsulphatase' has been applied to this enzyme (Robinson, Spencer & Williams, 1951). Arylsulphatase has been shown to be present in the tissues of higher animals (Neuberg & Simon, 1925; Rosenfeld, 1925; Hommerberg, 1931; Huggins & Smith, 1947) and also in neoplasms of the rat (Huggins & Smith, 1947).

Hitherto, no extensive study of the distribution of arylsulphatase among bacteria appears to have been made. An indication that this enzyme is present in some species of bacteria was obtained, however, by Barber, Brooksbank & Kuper (1951). These workers, while using sodium phenolphthalein monoglucuronide and sodium phenolphthalein diphosphate as substrates for the study of glucuronidase and phosphatase in staphylococci, kindly undertook to test the same organisms for arylsulphatase using, as substrate, potassium phenolphthalein disulphate synthesized by two of us (A.R.M. and L.Y.). Of the 160 strains of *Micrococcus pyogenes* and the seventy-five strains of coagulase-negative staphylococci tested by Barber *et al.* (1951), two showed faint phenolsulphatase activity. More marked arylsulphatase activity was shown, however, by an aerobic sporing bacillus encountered as a contaminant, and by a strain of *Salmonella schottmuelleri*.

In the present investigation, a wide range of bacterial species has been examined for the presence of arylsulphatase and some species have been found to contain the enzyme. The substrate used for testing for the presence of the enzyme was potassium phenolphthalein disulphate. With some bacterial species tests were also carried out using potassium 1-naphthylsulphate as substrate. The synthesis of potassium phenolphthalein disulphate does not appear to have been reported previously and an account of the method used to prepare the compound is given in the present paper, together with a description of some of its properties. A preliminary study of the separation of bacterial arylsulphatase has been made and an account is given of the preparation, from a strain of *Mycobacterium piscium*, of cell-free aqueous extracts with arylsulphatase activity.

A preliminary account of the work described in this paper has been published (Young, Morrison & Whitehead, 1952).

SUBSTRATES USED FOR THE STUDY OF BACTERIAL ARYLSULPHATASE

Potassium phenolphthalein disulphate

Synthesis of potassium phenolphthalein disulphate. Most of the methods used for the synthesis of arylsulphuric acids are based on a procedure developed by Verley (1901) in which chlorosulphonic

acid is added slowly to a chilled solution of an organic base in an anhydrous organic solvent. The addition product of the chlorosulphonic acid and the organic base is then allowed to react with the phenol, and the organic base salt of the arylsulphuric acid thus formed is converted to the potassium salt by treatment with aqueous potassium hydroxide. A number of organic bases and solvents have been used in this procedure, e.g. pyridine and carbon disulphide (Verley, 1901), pyridine and chloroform (Czapek, 1914), dimethyl- or diethyl-aniline and carbon disulphide (Burkhardt & Lapworth, 1926). In the present investigation it was found that potassium phenolphthalein disulphate can be prepared in fair yield by the above procedure using pyridine as the base and chloroform as a solvent.

A mixture of 12.5 ml. of dry pyridine and 12.5 ml. of redistilled CHCl_3 in a 100 ml. beaker (tall type) was cooled in an ice bath, and while the mixture was stirred, 5.0 ml. of chlorosulphonic acid were run in slowly down the side of the beaker from a tap funnel. To this solution were added quickly, with stirring, 8.0 g. of phenolphthalein in 15 ml. of CHCl_3 . The ice bath was then removed and the contents of the beaker were stirred for 2 hr., after which they were allowed to stand at room temperature overnight. The beaker was then placed in an ice bath and while its contents were stirred, 80% aqueous KOH solution (w/v) was added until the mixture was just alkaline to litmus (14 ml. of the KOH solution were required). The mixture was stirred for 10 min. after the addition of the KOH solution had been completed and it was then poured, with stirring, into 150 ml. of absolute ethanol. The precipitate which formed was allowed to settle in the refrigerator and was then separated by centrifuging. The solid material was then extracted on a water bath with three 100 ml. portions of a mixture of 80% ethanol and 20% water (v/v). During this process the mixture was kept just alkaline to phenolphthalein by the addition of a few drops of KOH solution. The extracts were cooled separately to room temperature and were then cooled in an ice bath. A heavy precipitate formed in the first extract and was separated by filtration. The filtrate was combined with the second and third extracts, 900 ml. of absolute ethanol were added, and the precipitate which formed was filtered off. The combined precipitates were dissolved in a minimum of water and the compound was salted out by the addition, with vigorous stirring, of 80% aqueous KOH solution (w/v). The potassium phenolphthalein disulphate was removed by filtration on a sintered-glass filter, washed with absolute ethanol, and dried. The product weighed 10.5 g. and this corresponded to a yield of 64% based on the amount of phenolphthalein used. The compound was crystallized twice from water and was then dried over P_2O_5 in *vacuo* at room temperature. The product gave negative tests for phenolphthalein, inorganic sulphate, chloride, carbonate and bicarbonate. It gave strong positive tests for phenolphthalein and inorganic sulphate after it had been boiled in N-HCl solution for 1 min. (Found: C, 36.7; H, 2.9; S, 9.8; K, 17.0. $\text{C}_{20}\text{H}_{13}\text{O}_{11}\text{S}_2\text{K}_3 \cdot 2\text{H}_2\text{O}$ requires C, 37.1; H, 2.6; S, 9.9, K, 18.1%. Micro-analyses by Drs Weiler and Strauss, Oxford.) The compound was very soluble in water and was almost insoluble in ethanol. It was obtained as fine colourless needles when precipitated from aqueous solution by the

addition of ethanol, and as rectangular plates when crystallized from water.

Gravimetric determination of phenolphthalein and sulphate liberated by acid hydrolysis of the compound. Phenolphthalein is almost insoluble in dilute HCl solution, and it was thus possible to carry out a gravimetric determination of the amount of phenolphthalein liberated on complete hydrolysis of the potassium phenolphthalein disulphate obtained by the procedure just described. The amount of sulphate liberated on hydrolysis was determined gravimetrically as BaSO_4 at the same time. To a solution of 0.06429 g. of the compound dissolved in 1 ml. of water in a small Pyrex filter beaker (fitted with a sintered-glass filter plate) was added 1 ml. of 2N-HCl. The vessel and its contents were heated in a boiling-water bath for 30 min. and then cooled in an ice bath. The crystalline precipitate was filtered off on the sintered-glass plate and washed with three 2 ml. portions of water. The filtrate and washings were collected. The precipitate, after it had been dried to constant weight at 105° , weighed 0.03182 g. It melted at $256-257^\circ$, and when it was mixed with phenolphthalein the melting point was unchanged. The sulphate present in the filtrate and washings yielded 0.04674 g. BaSO_4 . Colorimetric analysis of the filtrate and washings obtained in a similar experiment showed that the phenolphthalein content was negligible (<0.00005 g.). The phenolphthalein and sulphate (as SO_4) liberated corresponded to 49.5 and 29.9%, respectively, of the compound hydrolysed; potassium phenolphthalein disulphate dihydrate requires 49.2% phenolphthalein and 29.7% sulphate.

Colorimetric determination of phenolphthalein liberated by hydrolysis of the compound. In all experiments other than that just described, a colorimetric method was used to determine phenolphthalein liberated by the acid or enzymic hydrolysis of potassium phenolphthalein disulphate. The procedure was as follows. To 5 ml. of the acid (or buffer) solution containing phenolphthalein, NaOH was added from a microburette until the solution was just pink (the concentration of the NaOH solution used was such that the volume required did not exceed 1 ml.). After water had been added to bring the volume of the solution to 6 ml., 4 ml. of buffer solution, pH 10.4 (glycine-NaOH-NaCl, prepared as described by Talalay, Fishman & Huggins (1946)) was added. The red colour of the solution (after dilution, if necessary) was then measured in a Spekker photoelectric absorptiometer (Adam Hilger Ltd.) using an Ilford no. 605 filter. The amount of phenolphthalein present was then calculated by reference to a standard curve obtained using known amounts of phenolphthalein. By this means it was possible to determine as little as $10\mu\text{g.}$ of phenolphthalein in the original solution.

Experiments were carried out in which 1 ml. portions of a 0.01M-potassium phenolphthalein disulphate solution were heated in a boiling-water bath with 4 ml. portions of either 0.125N-HCl or 1.25N-HCl. Under these conditions it was found that the compound was completely hydrolysed in 15 min. in 0.1N-HCl and in 5 min. in N-HCl.

Similar experiments were carried out at 37° , and under these conditions it was found that in 24 hr. 7.8% of the phenolphthalein was liberated in 0.1N-HCl and 86.4% in N-HCl.

Enzymic hydrolysis of potassium phenolphthalein disulphate. Takadiastase, a commercial enzyme preparation from *Aspergillus oryzae*, shows arylsulphatase activity, and according to Huggins & Smith (1947) the enzyme has an

optimum pH of 6.12 at 37° with a substrate of potassium 4-nitrophenylsulphate. In order to study the behaviour of potassium phenolphthalein disulphate on enzymic hydrolysis, experiments were carried out in which takadiastase was allowed to act on the compound at 37° . The enzyme solution was prepared by extracting 2.5 g. of takadiastase diluted with talc (Parke Davis Co. Ltd.) with 50 ml. of water at room temperature. This gave a solution of takadiastase containing 0.37% solid matter, and 1 ml. portions of this were heated in a water bath at 37° with 1 ml. of 0.01M-potassium phenolphthalein disulphate solution and 3 ml. of citrate-phosphate buffer, pH 6.2 (McIlvaine series; Britton, 1942). The amounts of phenolphthalein liberated were determined colorimetrically and the results obtained are shown in Table 1. No liberation of phenolphthalein took

Table 1. *Hydrolysis of potassium phenolphthalein disulphate by takadiastase*

(1 ml. 0.01M substrate, 1 ml. 0.37% takadiastase, 3 ml. citrate-phosphate buffer, pH 6.2, at 37° .)

Time (hr.)	Phenolphthalein liberated ($\mu\text{g.}$)	Phenolphthalein liberated (%)
4	23	0.73
	22	0.69
8	52	1.64
	51	1.60
16	96	3.02
	98	3.08
24	143	4.50
	140	4.40

place in control experiments in which enzyme solution which had been heated in a boiling-water bath for 15 min. was allowed to act on the substrate for 24 hr. under the conditions just described.

Potassium 1-naphthylsulphate

Synthesis of potassium 1-naphthylsulphate. This compound was synthesized by the method of Feigenbaum & Neuberg (1941) using dimethylaniline as the organic base in the reaction. The potassium salt was purified by converting it to the *p*-toluidine salt by the procedure described by Barton & Young (1943), recrystallizing this compound, and then converting it back to the potassium salt. The product gave negative tests for free naphthol (see below) and inorganic sulphate. It gave positive tests for naphthol and inorganic sulphate after it had been heated with N-HCl in a boiling-water bath for 1 min. (Found: total sulphate S, 12.4. Calc. for $\text{C}_{10}\text{H}_7\text{O}_4\text{SK.}$ 12.2%.)

Detection of 1-naphthol liberated by hydrolysis of potassium 1-naphthylsulphate. 1-Naphthol was detected by the red colour it gives in alkaline solution with diazotized sulphanilic acid. The test was carried out by making the solution alkaline with NaOH solution and then adding freshly prepared diazo reagent (1 vol. of 0.8% NaNO_2 solution mixed with 9 vol. of a 0.25% solution of sulphanilic acid in N-HCl).

Stability of the substrates under conditions used for bacteriological tests

In the present investigation experiments were carried out in which bacteria were allowed to grow at 37° for 7 days in media containing potassium

phenolphthalein disulphate or potassium 1-naphthylsulphate. In the tests with *Actinomyces* the period of growth was 14 days. It was necessary, therefore, to determine whether any breakdown of the substrates took place under these conditions apart from that produced by the bacteria.

Into each of a series of glass-stoppered test tubes were placed 3 ml. citrate-phosphate buffer, pH 6.0, 7.0 or 8.0, 1 ml. of 0.01M-potassium phenolphthalein disulphate or 0.01M-potassium 1-naphthylsulphate, and 1 ml. of 0.1% 'Merthiolate' solution (Eli Lilly and Co. Ltd.) as a preservative. The tubes were placed in an incubator at 37° and at intervals up to 28 days, tubes were removed and their contents tested for the presence of phenolphthalein or 1-naphthol. No evidence of the breakdown of the substrates was obtained even after 28 days, i.e. after a period twice as long as the longest period used in the bacteriological tests.

As the media to which the substrates were added were sterilized by autoclaving at 15 lb./sq.in. pressure for 20 min., it was also necessary to determine whether any liberation of phenolphthalein or 1-naphthol occurred during this process. Tests on media with pH 7.0-7.6 containing 0.001M-potassium phenolphthalein disulphate or 0.001M-potassium 1-naphthylsulphate which had been sterilized in this way gave no evidence that hydrolysis of the substrates had taken place. Furthermore, there was no indication of hydrolysis after 2-3 months storage of the sterilized media at 4°. No detectable loss of the substrates occurred when their solutions were sterilized by Seitz filtration.

THE DISTRIBUTION OF BACTERIAL ARYLSULPHATASE

Apart from the investigations of Barber *et al.* (1951) on staphylococci, information appears to be lacking concerning the distribution of bacterial arylsulphatase. In the present work, various species of bacteria of medical interest have been examined for the occurrence of this enzyme. In order to test for the presence of the enzyme, the organisms were grown in a medium containing potassium phenolphthalein disulphate or potassium 1-naphthylsulphate, and the appearance of free phenolphthalein or 1-naphthol in the medium was taken as evidence of the production of arylsulphatase by the organism.

Methods

Preparation of media. An 0.01M solution of the substrate (potassium phenolphthalein disulphate or potassium 1-naphthylsulphate) in water was added to broth (pH 7.4-7.6) so as to produce a medium containing 1% meat extract (Lab Lemco, Oxo Ltd.), 1% peptone (Bacteriological Peptone, Evans Medical Supplies Ltd.), 0.5% NaCl, and 0.001M-substrate. The medium was distributed in 4 ml. portions in 6 × $\frac{3}{16}$ in. tubes and autoclaved for 20 min. at 15 lb./sq.in. pressure.

For the cultivation of anaerobes, hot sterile vaseline was added to the tubes immediately after their removal from the autoclave. When cool, the medium was inoculated in the usual manner. The vaseline seal was first melted by gentle heat and, after the medium had been inoculated, the vaseline was allowed to re-seal the tube.

For certain species, where it was needed for growth, 0.5 ml. sterile horse serum or blood was added to each tube after autoclaving, the tubes containing blood being brought to 100° for 2-3 min. and well shaken. In some experiments the medium was enriched with 0.5% glucose.

Detection of arylsulphatase production by organisms grown in media containing phenolphthalein disulphate. The strain to be tested was inoculated into three tubes of broth containing 0.001M-phenolphthalein disulphate, which were then incubated at 37°. One tube was tested for the presence of free phenolphthalein after 1 day, another after 3 days and the third after 7 days. Free phenolphthalein was detected by the addition of N-NaOH until maximum development of colour had taken place. Decolorization of phenolphthalein takes place in strongly alkaline solutions, and the NaOH solution was therefore added carefully drop by drop with shaking of the contents of the tube. The depth of colour which developed was assessed visually and recorded according to the scale shown in Table 2.

Detection of arylsulphatase production by organisms grown in media containing potassium 1-naphthylsulphate. Each strain examined with this substrate was inoculated into three tubes of broth containing 0.001M-potassium 1-naphthylsulphate and also into three tubes of broth without substrate. Control experiments using broth without substrate were carried out because it was found that faintly positive diazo reactions were obtained after growth had occurred. After incubation at 37° for 1 day a tube containing substrate and a control tube were removed and tested by means of the diazo reaction. The remaining tubes were tested after incubation for 3 and 7 days. The diazo test was carried out by adding to each tube 1 ml. of 2N-NaOH and 0.5 ml. of freshly prepared diazo reagent (1 ml. 0.8% NaNO₂ and 9 ml. 0.25% sulphanilic acid dissolved in N-HCl). The tubes were shaken and the difference in depth of colour between the tube containing naphthylsulphate and the control tube was assessed visually and recorded according to the scale shown in Table 2.

Sources of the strains studied. The strains examined were obtained from the following sources: the Stock Collection, Department of Bacteriology, St Thomas's Hospital Medical School; the Stock Collection and routine cultures, Louis Jenner Laboratory, St Thomas's Hospital; the Salmonella Reference Laboratory, Public Health Laboratory Service, Colindale; the National Collection of Type Cultures, Colindale. A collection of strains from cases of infantile gastro-enteritis was obtained from Dr G. S. Udall, St Thomas's Hospital Medical School.

The majority of the strains of salmonellae were inoculated directly from Dorset's egg medium. Strains of haemolytic streptococci, clostridia and corynebacteria were subcultured from Robertson's meat medium. The remainder were inoculated from nutrient or blood agar.

Results

Tests with potassium phenolphthalein disulphate as substrate. The number of strains tested with phenolphthalein disulphate as substrate was 212, and of these, fifty-four were found to show varying degrees of arylsulphatase activity. Among the organisms tested, the enzyme was found to be present in certain species of salmonellae and mycobacteria. For this reason the salmonellae and related bacteria

were studied more extensively than the other groups. The examination of large numbers of mycobacteria, many of which require special methods for their cultivation, was considered to be outside the scope of the present work and it is being made part of a separate investigation (Whitehead, Wildy & Engbaek, to be published).

Among seventy-nine strains of salmonellae, forty-seven strains belonging to fourteen species were found to give positive tests for the presence of arylsulphatase. No positive strains could be detected among nine strains of *Salm. typhosa*. Of the paratyphoid bacteria, all of the eleven strains of *Salm. paratyphi* tested, eight of twelve strains of *Salm. schottmuelleri*, and one of ten strains of *Salm. hirschfeldii* were found to give strongly positive tests. The strains of *Salm. paratyphi* showed somewhat weaker activity than the arylsulphatase-positive strains of the other paratyphoid bacteria. The four negative strains of *Salm. schottmuelleri* belonged to phage Types 1 and 2, and six of the positive strains to phage Types 3a, 3a1 and 3b. Unlike the paratyphoid organisms, the twenty-seven positive strains found among thirty-seven strains of other species of salmonellae showed weak arylsulphatase activity. Among the related bacteria, no activity was detectable among twenty strains of *Shigella*, twenty-nine strains of *Escherichia*, *Aerobacter* and *Klebsiella*, and twenty-one strains of *Proteus*.

The ten strains of mycobacteria examined included four strains originally isolated from cold-blooded animals. Of these four strains, two of *Mycobact. piscium* and one of *Mycobact. ranae* showed considerable activity, while a strain of *Mycobact. chelonae* was less active. Of the saprophytic acid-fast bacteria, two strains each of *Mycobact. phlei*, *Mycobact. smegmatis* and *Mycobact. stercoris* were tested. One strain of each species gave negative tests for arylsulphatase, and one strain of each species showed weak activity.

The following organisms, in addition to those already mentioned, gave no evidence of the production of arylsulphatase (where more than one strain was examined the number is shown in brackets): *Actinomyces* (anaerobic strain from human actinomycosis); *Bacillus anthracis*, *B. subtilis* (3), other Gram-positive sporing bacilli (5); *Clostridium perfringens*, *Cl. septicum*, *Cl. sporogenes*, *Cl. tetani*, *Cl. tetanomorphum*; *Corynebacterium diphtheriae* var. *gravis*, *C. diphtheriae* var. *intermedius*, *C. diphtheriae* var. *mitis* (3), *C. pseudodiphtheriticum*, *C. xerosis*; *Diplococcus pneumoniae*; *Gaffkya tetragena*; *Haemophilus influenzae*, *Haem. pertussis*; *Micrococcus lysodeikticus*; *Neisseria meningitidis* (2); *Pasteurella pestis*; *Pseudomonas aeruginosa* (4); *Sarcina*; *Serratia marcescens*; *Streptococcus faecalis* (2), haemolytic streptococci (7), non-haemolytic

streptococci (3), *Strep. viridans* (3); *Vibrio comma* (2).

Tests with potassium 1-naphthylsulphate as substrate. A number of tests for arylsulphatase were carried out using potassium 1-naphthylsulphate as substrate in order to find whether the results obtained would be similar to those from experiments in which phenolphthalein disulphate was used as substrate. Included in these tests were the majority of the salmonella strains which were negative or weakly positive, and a few which were strongly positive when tested on a substrate of phenolphthalein disulphate. In all, fifty-seven strains of this group were tested with a substrate of potassium 1-naphthylsulphate. The results are included in Table 2, and they show that there is a broad measure of agreement in the results obtained with the two compounds, although in some instances hydrolysis of the substrate was detectable earlier when potassium 1-naphthylsulphate was used. In addition to the salmonella strains, the following sixteen strains from other genera were tested with potassium 1-naphthylsulphate as substrate and they yielded results in accordance with those obtained with phenolphthalein disulphate: *Aerobacter aerogenes*, *B. anthracis*, *B. subtilis*, *Esch. coli* (2), *Klebsiella pneumoniae*, *Micrococcus epidermidis*, *M. pyogenes* var. *aureus*, *Mycobact. piscium*, *Past. pestis*, *Ps. aeruginosa*, *Proteus vulgaris* (2), *Shigella sonnei*, *Strep. pyogenes*, *V. comma*.

During the examination of some strains in media containing potassium 1-naphthylsulphate it was observed that after incubation for 24 hr. or more there was a partial clearing of the turbidity of the cultures. It was noticed that this only occurred with strains which produced arylsulphatase and never with those which did not.

Experiments using a colourless medium. In an attempt to detect slight degrees of hydrolysis of phenolphthalein disulphate by bacteria, trials were made with this compound in a colourless medium consisting of 1% peptone (Bacteriological Peptone, Evans Medical Supplies Ltd.) and 0.5% sodium chloride in water. The substrate concentration was 0.001M. This medium was used for the examination of sixty-seven strains from various genera tested using the other medium. It was found that although slight hydrolysis of phenolphthalein disulphate could more easily be detected in the colourless medium, this advantage was outweighed by the poorer growth which occurred with many organisms. No additional arylsulphatase-producing strains were detected with the colourless medium.

Experiments with a medium containing 0.5% glucose. In order to determine whether the production of arylsulphatase is affected by the enhanced growth which many bacterial species show in media containing glucose, two strains known to

produce the enzyme were grown in nutrient broth containing 0.001M-phenolphthalein disulphate and, in addition, 0.5% glucose. No arylsulphatase activity could be detected after incubation for 7 days, although the organisms showed more vigorous growth than was observed in the absence of glucose. All the salmonellae which had been shown to produce the enzyme were therefore examined in broth containing 0.5% glucose and the

substrate, and none showed any trace of arylsulphatase activity. Under the same conditions no activity could be detected in any of six strains of *Mycobact. phlei*, *Mycobact. smegmatis* and *Mycobact. stercoris*, whereas the four strains of *Mycobact. piscium*, *Mycobact. ranae* and *Mycobact. chelonae* showed no impairment of arylsulphatase activity when grown in broth containing 0.5% glucose. Twenty strains from various genera which were

Table 2. Results of testing seventy-nine strains of salmonellae for arylsulphatase activity using potassium phenolphthalein disulphate (I) and potassium 1-naphthylsulphate (II) as substrates

(Substrate I. Colour on addition of alkali to the medium: \pm , faint tinge of pink; +, pale pink; ++, pink; +++, red; +++++, deep red; -, no change in colour. Substrate II. Colour difference between control and medium containing substrate on addition of alkali and diazo reagent: \pm , just discernible; ++, moderate; +++++, marked; -, no difference. NT=strain not tested.)

Species of Salmonella	No. of strains tested	Arylsulphatase activity					
		1 day		3 days		7 days	
		Substrate I	Substrate II	Substrate I	Substrate II	Substrate I	Substrate II
<i>Salm. typhosa</i>	9	-	-	-	-	-	-
<i>Salm. paratyphi</i>	1	-	++	++	++++	++++	++++
	1	-	++	++	++	++++	++++
	3	\pm	NT	++	NT	++++	NT
	4	\pm	NT	+++	NT	++++	NT
	1	+	NT	++++	NT	++++	NT
	1	++	NT	++++	NT	++++	NT
	2	++	NT	+++	NT	++++	NT
<i>Salm. schottmuelleri</i>	2	-	-	-	-	-	-
	2	-	-	-	\pm	-	-
	5	++	NT	++++	NT	++++	NT
	1	++	++	++++	++++	++++	++++
<i>Salm. hirschfeldii</i>	1	++	++++	++++	++++	++++	++++
<i>Salm. hirschfeldii</i>	9	-	++++	++++	++++	++++	++++
<i>Salm. sp.</i> (Type Aberdeen)	1	-	-	\pm	-	+	\pm
<i>Salm. abortusovae</i>	1	-	-	-	-	-	-
<i>Salm. moribundans</i>	1	-	-	-	-	\pm	-
<i>Salm. choleraesuis</i>	1	-	-	-	-	-	-
<i>Salm. sp.</i>	1	-	-	-	\pm	\pm	++
	1	-	\pm	-	\pm	+	++
	1	-	-	-	-	-	\pm
	1	-	-	-	-	++	++
	1	-	-	-	-	\pm	\pm
<i>Salm. enteritidis</i>	1	-	-	++	\pm	++	++
	1	-	NT	-	NT	\pm	NT
	2	-	-	-	-	\pm	-
	1	-	-	-	-	\pm	\pm
<i>Salm. sp.</i> (Type London)	1	-	-	++	-	+	-
<i>Salm. sp.</i> (Type Newington)	1	-	-	\pm	-	\pm	\pm
<i>Salm. sp.</i> (Type Newport)	1	-	NT	\pm	NT	++	\pm
	2	-	-	-	-	\pm	\pm
	1	-	-	-	-	\pm	-
<i>Salm. sp.</i> (Type Poona)	1	-	-	-	-	+	-
<i>Salm. sp.</i> (Type Potsdam)	1	-	-	-	-	\pm	-
<i>Salm. sp.</i> (Type Senftenberg)	1	-	-	-	-	\pm	-
<i>Salm. sp.</i> (Type Thompson)	1	-	\pm	-	-	++	\pm
	1	-	NT	-	NT	+	NT
	1	-	-	-	-	-	-
	2	-	-	-	-	-	\pm
<i>Salm. typhimurium</i>	1	-	-	-	-	-	++
	1	-	NT	-	NT	-	NT
	1	-	-	-	-	-	-
	2	-	-	-	-	\pm	\pm
	2	-	-	-	-	\pm	-

negative when tested in nutrient broth showed no arylsulphatase activity when grown in glucose broth.

Experiments under anaerobic conditions. Two strains of *Salm. schottmuelleri* and one of *Salm. paratyphi* which produced arylsulphatase when grown aerobically were cultured under anaerobic conditions by the methods described earlier. In each case visible growth was less than when these organisms were grown aerobically, but arylsulphatase production appeared to be only slightly diminished.

SEPARATION AND PROPERTIES OF BACTERIAL ARYLSULPHATASE

In order to study the properties of bacterial arylsulphatase, experiments designed to separate the enzyme from bacteria were undertaken. As a result of this work it was found possible to prepare cell-free aqueous solutions of arylsulphatase and these have been used for preliminary studies of the properties of the enzyme.

The organism from which arylsulphatase was separated was a subculture of *Mycobact. piscium*, N.C.T.C. No. 2291. This was obtained originally about 10 years ago from the National Collection of Type Cultures, and since then the strain has been maintained by monthly subculture on nutrient agar slopes. The original strain was isolated from the diseased roe of a halibut by Griffith (1930). This organism was selected for the present investigation because its arylsulphatase activity was high compared with that of other organisms which had been tested. Additional advantages attending its use were its lack of pathogenicity to man and the fact that its form of growth on a liquid medium is such that it can be harvested easily. At 37° growth of the organism reaches a maximum after about 5–7 days and on liquid media it takes the form of a moderately tenacious yellowish grey pellicle with little sub-jacent turbidity or deposit, thus rendering harvesting by filtration a simple procedure.

Growth and harvesting of the bacteria. The organism was grown in 4 oz. 'medical flat' bottles loosely plugged with cotton wool, each containing 30 ml. nutrient broth containing 0.001 M-potassium phenolphthalein disulphate. Inoculation was carried out by carefully floating on to the surface of the medium a portion of surface pellicle (approx. 1.5 cm. in diameter) from a 3–5-day broth culture. The bottles were then incubated in a horizontal position so as to expose the greatest surface area of medium for growth of the organisms. The growth was harvested after 5–7 days incubation at 37°, by which time the surface of the medium was almost completely covered by the pellicle. The bacterial mass was separated by filtration on a Büchner funnel using a no. 1 Whatman paper and it was washed with about 100 ml. of distilled water for each bottle whose contents were filtered. Most of the excess moisture was removed by suction on the filter.

Extraction of the enzyme from the bacterial mass. Examination of the culture filtrate showed that its arylsulphatase activity was low. Attempts were made, therefore, to obtain active preparations of the enzyme by extracting the bacilli. No arylsulphatase activity was found in extracts obtained by hand-grinding moist bacilli or acetone-dried bacilli in an agate mortar with saline-phosphate buffer at pH 7.0. In each case tests showed that the activity remained in the residue of the ground bacilli. Active preparations were obtained, however, when the technique of mechanical grinding of bacteria recommended by Dockstader & Halvorson (1950) was employed. The procedure which was developed for the preparation of cell-free aqueous solutions of the enzyme was as follows.

The bacterial mass which had been separated by filtration on a Büchner filter and washed with distilled water was dried by repeated washing with acetone followed by suction. The dried material was scraped off the filter paper and weighed. It was then mixed with twice its weight of Hyflo Super-Cel (Johns-Manville Co. Ltd.) which had previously been washed three times with distilled water and dried. The mixture was placed in a homogenizer (Potter, 1945) and twice its volume of distilled water was added. The tube containing the mixture was immersed in an ice bath for 15 min. while its contents were ground at about 1500 rev./min. The viscid creamy material obtained by grinding was suspended in ice-cold distilled water (1 ml./5 mg. of acetone-dried bacilli) and centrifuged at 1800 g for 30 min. The opalescent supernatant liquid was decanted and filtered through a Gradocol collodion membrane (average pore diameter, 0.52 μ .) in an Elford ultrafilter to remove any remaining bacterial cells. The filtrate obtained was clear and showed only a very faint yellowish tinge. Dry weight determinations on two filtrates obtained in this way gave values of 1.42 and 1.57 mg./ml.

By means of the procedure just described it was possible to obtain cell-free solutions of arylsulphatase from bacilli grown in the presence of phenolphthalein disulphate. Even when the procedure was modified by the omission of phenolphthalein disulphate from the medium the final solution obtained was found to contain the enzyme. Arylsulphatase was also separated from bacilli grown from an inoculum of organisms which had never been in contact with phenolphthalein disulphate.

Optimum pH of the enzyme. Experiments were conducted in which the enzyme was allowed to act on potassium phenolphthalein disulphate at various pH values ranging from 3.0 to 8.0. The experiments were carried out in glass-stoppered Pyrex test tubes into each of which was placed 3.0 ml. of citrate-phosphate buffer (McIlvaine series; Britton, 1942), 1.0 ml. of 0.01 M-potassium phenolphthalein disulphate and 1.0 ml. of unbuffered enzyme preparation. After 6 hr. in the water bath at 37° the tubes were transferred to an ice bath and the free phenolphthalein was determined by the method

described earlier in the present paper. The results obtained from duplicate experiments with each of two enzyme preparations are shown in Table 3. Experiments in which the pH values of the buffer-substrate-enzyme systems were determined electrometrically at the beginning and the end of the period of incubation revealed no significant change in pH. No detectable liberation of phenolphthalein occurred in 6 hr. at 37° in control experiments in which water was used in place of enzyme solution at each pH value studied, or in which enzyme solution which had been heated in a boiling-water bath for 20 min. was allowed to act on the substrate at pH 6.3.

Table 3. *Optimum pH of arylsulphatase present in cell-free extracts of a strain of Mycobacterium piscium*

(3.0 ml. citrate-phosphate buffer, 1.0 ml. 0.01 M-potassium phenolphthalein disulphate, and 1.0 ml. enzyme solution at 37° for 6 hr.)

pH	Phenolphthalein liberated ($\mu\text{g.}$)	
	Enzyme preparation A	Enzyme preparation B
3.0	2	—
4.0	12	—
5.0	50	—
5.6	—	21
6.0	59	23
6.3	—	24
6.7	—	20
7.0	47	16
8.0	9	—

Table 4. *Optimum substrate concentration of arylsulphatase present in cell-free extracts of a strain of Mycobacterium piscium*

(3.0 ml. citrate-phosphate buffer pH 6.3, 1.0 ml. potassium phenolphthalein disulphate solution, and 1.0 ml. enzyme solution at 37° for 6 hr.)

Substrate concn. in system (M)	Phenolphthalein liberated ($\mu\text{g.}$)
0.0005	13
0.0010	19
0.0020	21
0.0030	21
0.0040	20
0.0050	20

From the results given in Table 3 it appears that under the conditions employed the optimum pH of the arylsulphatase present in the extracts lay in the vicinity of 6.3.

Optimum substrate concentration. In order to determine the optimum substrate concentration a series of experiments was carried out in each of which 3.0 ml. of citrate-phosphate buffer pH 6.3 and 1.0 ml. of enzyme solution were heated for 6 hr. at

37° with 1.0 ml. of potassium phenolphthalein disulphate solution and the phenolphthalein liberated was determined colorimetrically by the method already described. The phenolphthalein disulphate solutions used were such that the concentrations of substrate in the system ranged from 0.0005 to 0.005 M at the beginning of the experiment. The results obtained are shown in Table 4 from which it appears that the maximum velocity of arylsulphatase action occurred with a substrate concentration of 0.002–0.003 M.

DISCUSSION

As the present investigation involved the testing of many different bacteria for the presence of arylsulphatase, it was necessary first to devise a simple, sensitive test for the enzyme which could be applied in a medium suitable for the growth of a wide variety of organisms. Various substrates have been used for the qualitative and quantitative study of plant and animal arylsulphatase. These include potassium indoxyl sulphate (Derrien, 1911; Neuberg & Wagner, 1925), potassium phenylsulphate (Neuberg & Kurono, 1923; Abbott, 1947), and potassium 4-nitrophenylsulphate (Neuberg & Wagner, 1925; Huggins & Smith, 1947), and while the present work was in progress, Robinson, Smith & Williams (1951) described the use of potassium 2-hydroxy-4-nitrophenylsulphate for the study of the enzyme. The use of these compounds has been based on the fact that on hydrolysis they yield phenolic products which can be detected or determined by their conversion to coloured compounds on treatment with suitable reagents. In applying this principle to the detection of bacterial arylsulphatase, consideration has to be given to the fact that culture media often have a yellow colour, and in consequence compounds which on hydrolysis yield phenols which are detected by their conversion to yellow products (e.g. potassium 4-nitrophenylsulphate) are unsatisfactory. In order to study bacterial phosphatase, Bray & King (1942) used phthalein phosphates as substrates and later they made a study of the distribution of phosphatase in micro-organisms using phenolphthalein diphosphate as substrate (Bray & King, 1943). Phenolphthalein offers a number of advantages as a chromogen, the chief of which are that the red-coloured form can be obtained merely by the addition of alkali, very small amounts of phenolphthalein are readily detected, and the colour reaction is not susceptible to interference by substances present in biological material. For these reasons phenolphthalein diphosphate has been used for work on phosphatases (Bray & King, 1943; Huggins & Talalay, 1945; Barber *et al.* 1951) and phenolphthalein glucuronide has been used for the

study of β -glucuronidase in mammalian tissues (Talalay *et al.* 1946) and in micro-organisms (Barber *et al.* 1951). These various considerations led us to use phenolphthalein disulphate as an agent for testing for arylsulphatase. Some tests were also carried out with a monosulphate, potassium 1-naphthylsulphate, as substrate, in order to determine whether the use of a different substrate would influence our conclusions concerning the distribution of arylsulphatase. The results obtained with this compound, however, were in general agreement with those obtained using phenolphthalein disulphate. As an agent for testing for arylsulphatase in bacteria, potassium 1-naphthylsulphate suffers from the disadvantage that it is necessary to run control experiments, for most organisms yield faintly positive diazo reactions after growth has occurred in the absence of the substrate.

The organisms tested for arylsulphatase activity in the present work did not include staphylococci or anaerobic cocci. The distribution of arylsulphatase among staphylococci has been investigated by Barber *et al.* (1951) and the distribution of the enzyme among anaerobic cocci has been studied in the course of an investigation by Prof. R. Hare & Dr M. P. L. Wildy of the Bacteriology Department of this School (to be published). A wide variety of organisms was examined in the present work, and the results suggest that the possession of arylsulphatase in readily detectable amounts is not common among bacteria. Arylsulphatase activity was found in certain species of salmonella and mycobacteria, but with the methods used it could not be detected in any of the other species tested. The possibility should not be overlooked that the examination of larger numbers of strains of a particular species might reveal the occurrence of further arylsulphatase-producing organisms. It must also be recognized that, whereas a positive finding is significant in an investigation of this type, a negative finding does not necessarily exclude the possibility that the organism produces the enzyme. Variation of the cultural conditions or the use of a more sensitive method of testing might reveal the presence of the enzyme in an organism with weak arylsulphatase activity. In this connexion it should be noted that tests carried out on organisms of the *Salmonella* group with a monosulphate, potassium 1-naphthylsulphate, revealed the occurrence of weak arylsulphatase activity in a few strains earlier than when phenolphthalein disulphate was used as the substrate, although in most cases the two tests were in agreement. Another possibility which has to be considered is that the phenolic compound liberated from the substrate by the action of the enzyme might be metabolized by the organism and thus escape detection. This question is under investiga-

tion by Whitehead, Wildy & Engback (to be published) in their study of the arylsulphatase activity of mycobacteria using phenolphthalein disulphate as substrate, but no evidence has so far been obtained suggesting that utilization of phenolphthalein by these organisms (whether they produce arylsulphatase or not) occurs to an extent likely to affect the detection of the enzyme. Whether this is generally true of other bacterial species has not been established.

From a biochemical standpoint considerable interest attaches to the separation of the enzyme and the study of its properties. Among the various bacteria studied, one of the most active producers of arylsulphatase was a strain of *Mycobact. piscium*. Examination of the culture medium of this organism grown in the presence or the absence of phenolphthalein disulphate suggested that the medium contained little or no arylsulphatase. When the organism itself was separated and dried with acetone, it was found possible to prepare cell-free aqueous extracts which contained much of the arylsulphatase activity originally present in the dried organisms. These extracts were used for studies of the optimum pH and optimum substrate concentration of the enzyme. These preliminary experiments on the separation and properties of bacterial arylsulphatase have paved the way for more extensive biochemical studies of the enzyme.

SUMMARY

1. A description is given of the synthesis and properties of potassium phenolphthalein disulphate.
2. Potassium phenolphthalein disulphate has been used as a substrate for testing for the presence of arylsulphatase (phenolsulphatase) in 212 strains from a wide range of bacterial species, and seventy-three of these strains have also been tested for the presence of this enzyme using potassium 1-naphthylsulphate as substrate. The results obtained with the two substrates are in general agreement.
3. Under the test conditions, arylsulphatase has been detected only in certain strains of salmonellae and mycobacteria.
4. Cell-free aqueous extracts containing arylsulphatase have been prepared from *Mycobacterium piscium*, N.C.T.C. 2291, and these have been used in a preliminary study of the properties of the enzyme.

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Microdetermination of Manganese in Biological Material by a Modified Catalytic Method

BY H. FORE AND R. A. MORTON

Biochemistry Department, University of Liverpool

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In investigating the biochemical roles of manganese it is desirable to employ a very sensitive but reasonably accurate method of estimating the element. Methods based on the detection of manganese lines in emission spectra require the presence of about 50 $\mu\text{mg.}$ of the element in a 10 mg. sample, and even when determinations are carried out in triplicate the accuracy obtainable is not great.

The periodate method, based on oxidation of the manganese to permanganate, which is determined by photoelectric colorimetry or spectrophotometry (Willard & Greathouse, 1917; Richards, 1930; Strickland & Spicer, 1949; Davidson & Capen, 1929; Mehlig, 1939, 1941; Kirk, Rosenfels & Hanahan, 1947; Rowland, 1939) is generally satisfactory. The main disadvantage of the periodate method is that the amount of tissue needed to provide the necessary minimum of manganese is quite often larger than that available. The colorimetric estimation of manganese by means of the Folin-Ciocalteu reagent (Abul-Fadl, 1948) requires at least 12 $\mu\text{g.}$ manganese.

Szebelledy & Bartfai (1936) described a catalytic method for the detection of manganese in amounts less than 1 $\mu\text{mg.}$, and Kun (1947) worked out a quantitative procedure, using photoelectric spectrophotometry. Manganous ions catalyse the oxidation of *NN*-diethylaniline by potassium metaperiodate (KIO_4) in aqueous solution, and the progress of the oxidation can be followed by measuring the increasing absorption of light at 470 $\text{m}\mu$. Kun used a medium buffered at pH 7.0 and measured the extinction exactly 200 sec. after mixing the reagents. He found no interference by ions of other metals. Each test required at least 4 $\mu\text{mg.}$ of manganese, but Kun found it necessary to make many readings so as to eliminate the effect of what appeared to be random variations.

The catalytic method appeared to be very promising, but a considerable study of all the variables was necessary before it could be applied with confidence to the problems of determining manganese in very small amounts of tissues. The final procedure adopted will first be described and then discussed.

EXPERIMENTAL

Reagents

All reagents must be stored in Pyrex vessels.

Glass-distilled water. Distilled water from the usual type of laboratory still is redistilled from all-glass Pyrex apparatus.

Hydrochloric acid. Spray-free hydrogen chloride is passed, via a wax-coated funnel, into glass-distilled water in a wax-lined beaker immersed in ice. The acid (approx. 16% w/v) is stored in wax-lined bottles.

Buffer solution (pH 7.0). This is prepared by mixing solution *a* (40 ml.) and solution *b* (approx. 30 ml.) and making up to 100 ml. Solution *a* consists of citric acid (A.R., 111 g.) and 'pure' conc. HCl (29 ml.) in 2.5 l. Solution *b* is prepared as follows: approx. 2N-NaOH (A.R. carbonate-free) is standardized and boric acid (A.R., 122 g.) is dissolved in exactly 2.5 equiv. of the alkali solution and the whole made up to 2.5 l.

Diethylaniline hydrochloride solution. Diethylaniline (0.12 g.) is weighed accurately and dissolved in 1.4 equiv. of 0.1N-HCl. The solution is made up to 1 l. (0.0008M).

Potassium periodate solution. KIO_4 solution (0.01M) is made by dissolving 2.3 g. of the A.R. salt in 800 ml. of hot water, cooling and diluting to 1 l. in a graduated flask.

Manganese solution. A.R. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was used for preparing the standard manganese solution.

Method

A 'translucent Vitreosil' crucible is cleaned with abrasive soap (Briz), boiled in conc. HCl, washed in glass-distilled water and heated for 30 min. at 650°. It is cooled in a desiccator, then weighed and after inserting the fresh tissue sample, weighed again. The tissue is dried to constant weight at 110° and is then ashed at not over 650° in a silica-lined muffle furnace. Glass-distilled water may be added to the cooled residue to assist removal of the last traces of carbon (this step is empirical but effective). The crucible is cooled and weighed, platinum-tipped crucible tongs being used throughout.

A measured volume of the 16% HCl is added to the crucible; this is placed inside a 100 ml. beaker and left on a steam bath until the HCl has evaporated. Buffer solution (volume depending on the amount of Mn in the sample) is added to the crucible which is then placed on the steam bath for 20–30 min. (2 ml. of buffer is used for samples containing up to 50 $\mu\text{mg.}$ including the Mn contaminating the HCl. With larger samples proportionately more buffer is added. When the amount of Mn is very large the crucible is extracted with a known volume of HCl instead of with buffer and the solution washed into a graduated flask of suitable size. After making up to volume, a portion of the solution is dispensed into a clean crucible, evaporated to dryness and then taken up in buffer as described above.) The beaker is then removed and allowed to cool, protected from dust. With the aid of a small Pyrex funnel and a wash-bottle (Pyrex) with the shortest possible rubber connexion, the contents of the crucible are washed into a graduated flask of a capacity 2.5 times the volume of buffer taken. Pyrex graduated flasks should be used if obtainable.

Four such buffered solutions of ash are made up, together with a blank consisting of diluted buffer alone and a standard consisting of diluted buffer containing 4.00 $\mu\text{mg. Mn/ml.}$

These six solutions, the reagents, six 1 ml. pipettes and 36 Pyrex test tubes (9 × 75 mm. with rim), together with other necessary apparatus, are left in the instrument room overnight. Within 24 hr. of making the buffered ash solutions and at a time when the room temperature is fairly stable three 1 ml. portions of each buffered solution are dispensed into test tubes and 1 ml. portions of KIO_4 solution into the remaining 18 tubes. Taking the solutions in the order in which they were pipetted (three blanks, four × three buffered ash solutions, then three standards) 0.5 ml. of diethylaniline hydrochloride solution is added from a graduated pipette to the buffered solution. The mixture is immediately poured into the KIO_4 solution, the time at that instant noted on a stop-clock and the reaction mixture agitated by repeatedly pouring from one tube to the other. The solution, in which the colour is now developing, is transferred to a clean dry absorption cell (1.0 cm.) and placed in the photoelectric spectrophotometer. The *E* value of the solution is read exactly 60 sec. after mixing.

The cell containing the reaction mixture is rinsed out four to six times with glass-distilled water, three times with redistilled absolute ethanol and three times with redistilled ether. It is then allowed to dry with a current of dust-free air blowing through it and another cell, used in the previous measurement, is used for the next reaction mixture. At the end of the run the pipettes used for the six buffered solutions are washed out in the manner described for the cells. The test tubes are all cleaned carefully before use. New ones are first cleaned with abrasive soap, but the procedure used for cleaning the tubes for each run is as follows: the tubes are filled with HCl (1 vol. conc. acid + 1 vol. water) and allowed to stand for a few minutes. The acid is poured out and the tubes rinsed three times with tap water, three times with distilled water and three times with glass-distilled water. The tubes are then drained, dried in an electric oven and stored away from dust.

The mean of the three extinction values (*E*) for each buffered solution is obtained. The means for the blank and standard are used to fix the standard straight line from which the Mn concentrations corresponding to the *E* values for the four test solutions are interpolated. From the Mn concentration in the reaction mixture the amount of Mn derived from each sample can be calculated and hence the Mn concentrations in fresh tissue, dried tissue and ash.

RESULTS

The above procedure was developed as a result of an exhaustive investigation of the reaction conditions. The experimental findings can be summarized as follows:

(a) A minimum of 1 $\mu\text{mg.}$ of manganese is required for a single determination, but owing to variability in the colour intensity produced, three determinations must be made for each sample examined. In practice the sample should contain at least 5 $\mu\text{mg.}$ of manganese. With the reagents used in this method, the extreme sensitivity (to 0.1 $\mu\text{mg.}$) claimed by Szebelledy & Bartfai in 1936 for a catalytic method of detecting manganese was not attainable in our hands.

(b) The manganese concentrations in the reaction mixture itself could be determined to $\pm 10\%$

Table 1. *Comparison of the catalytic and periodate methods*

Wt. of liver taken (g.)	Sample taken	Estimated Mn (μ g.)	Total Mn from liver (μ g.)	Mn content (p.p.m.)
Periodate method				
9.98	—	27.2	27.2	2.73
10.26	—	27.3	27.3	2.67
				Mean 2.70 \pm 1 %
Catalytic method				
10.24	1/100	0.303	30.3	2.96
	1/100	0.274	27.4	2.67
10.36	1/100	0.262	26.2	2.53
	1/100	0.265	26.5	2.56
				Mean 2.69 \pm 10 %

(deviation from the mean) over the range 0.4–4.0 μ mg./ml. and this permitted similar accuracy in determinations of the manganese contents of moderately large samples of tissue. With smaller samples (containing less than 10 μ mg. Mn) reproducibility was not quite so good, possibly owing to contamination, and the accuracy was about \pm 20 %. In Kun's experience the accuracy attainable for the determinations of manganese concentration over the range 0.4–40 μ mg./ml. was \pm 15 %.

(c) The rate of the reaction was directly proportional to the manganese concentration under the conditions already described. Kun obtained a logarithmic relationship but his experimental conditions differed considerably from ours.

(d) The possibility of interference was studied for the following ions: Cr^{+++} , Co^{++} , Cu^{++} , Fe^{++} , Ni^{++} , Mg^{++} , K^+ , Hg^{++} , Sn^{++} , Pb^{++} and Zn^{++} . In each case phosphate ions were present in the buffer. The only effect noticed was that the first three ions exerted an inhibitory effect, but in no case was it shown at a concentration small enough to have relevance to the determinations on tissues.

(e) To obtain satisfactory results the pH must be kept constant to within 0.1 unit. The reagents need to be kept overnight in the instrument room and the actual determinations on the spectrophotometer must only be made when the room temperature is steady.

(f) By using diethylaniline hydrochloride solution containing excess hydrochloric acid, it was found that the reagent need not be freshly prepared, but should not be more than a fortnight old.

(g) It was important to adhere exactly to the procedure described for mixing the solutions, otherwise the intensities of absorption and the shape of the standard curve varied markedly.

(h) The addition of hydrochloric acid to the ash and subsequent evaporation to dryness is an essential step in ensuring that the ash is soluble in the buffer. It also serves to convert any Mn^{++++} or Mn^{+++} present in the ash to Mn^{++} . The hydrochloric acid contained a trace of manganese, even when

prepared as described (p. 595), and allowance had to be made for this in working out the results.

(i) Excellent agreement was obtained when the manganese content of ox liver was determined by both the catalytic method and the periodate method using large samples. The results of such an experiment are given in Table 1.

Table 2. *Comparison of the catalytic and periodate methods using small samples for the former and silica or platinum crucibles for both*

Wt. of liver (g.)	Estimated Mn (μ g.)	Mn content (p.p.m.)
Periodate method (silica crucibles)		
3.05	6.5	2.1
2.90	5.0	1.7
2.18	4.0	1.8
		Mean 1.9 \pm 11 %
Periodate method (platinum crucibles)		
1.90	4.5	2.4
2.11	4.7	2.2
		Mean 2.3 \pm 4 %
Catalytic method (silica crucibles)		
(mg.)	(μ mg.)	
2.47	6.2	2.5*
1.24	5.1	4.1*
1.18	0.6	0.5*
1.23	1.8	1.5*
		Mean 2.2 \pm 77 %*
Catalytic method (platinum crucibles)		
1.14	9.9	8.7
1.12	8.6	6.8
		Mean 7.8 \pm 13 %

* These results show that the method is only semi-quantitative for amounts of material as small as about 1 mg.

(j) Samples ashed below 650° in silica crucibles lost some manganese by combination with the silica and were therefore expected to give low results. When very small samples for determination by the catalytic method were ashed in platinum

crucibles, however, the results obtained were higher than those obtained by the periodate method using larger samples also ashed in platinum crucibles. The results obtained by the catalytic method using very small samples ashed in silica crucibles agreed well with those (presumably the true ones) obtained by the periodate method using platinum crucibles. This was presumably due to a fortuitous compensation for the loss due to combination with the silica by some unknown factor. Table 2 illustrates this point with the results obtained when the method was applied to a protein powder prepared from ox liver.

(m) When the sample examined (e.g. blood) contained a large excess of iron the reaction mixture was coloured green and the readings obtained at 470 m μ . were below those for the blank. Thus determination of manganese in such material by the catalytic method was not possible.

Mechanism of the reaction

No direct attempt has been made to elucidate the mechanism of the catalytic process, but the development of the method yielded some relevant information.

Table 3. Results of experiments with added manganese

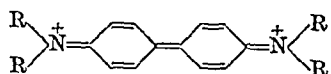
Sample of tissue (mg.)	Mn added (μ mg.)	Mn estimated (μ mg.)	Mn content (p.p.m.)	
			Estimated	Calculated
54.2	Nil	11.7	0.22	
54.2	Nil	10.7	0.20	
		Mean	0.21	
54.2	10.0	21.1	0.39	
54.2	10.0	25.2	0.46	
		Mean	0.43	0.39
54.2	20.0	29.8	0.55	
54.2	20.0	36.2	0.67	
		Mean	0.64	0.58
54.2	30.0	41.8	0.77	
54.2	30.0	38.6	0.71	
		Mean	0.74	0.76

This table also shows that, for samples containing less than 5 μ mg. of manganese, the deviation of the results from the mean is no longer within $\pm 20\%$. Kun observed good agreement between his catalytic method and a method based on the oxidation of manganese to MnO_4^- , but in making the comparison he used relatively large samples only.

(k) A puzzling phenomenon was observed in some calibration experiments. The usual procedure of adding known amounts of manganese to the ash of a sample and determining the manganese present was followed. The individual differences between calculated and observed manganese content bore no relation to the relative amount of added manganese and the observed values were, on the average, 10% higher. Table 3 gives the results of one such experiment in which eight samples of an acid solution of an ox eye-tissue (sclera) ash were evaporated in separate crucibles, known amounts of manganese being added to six of them. The manganese in all the crucibles was then determined.

(l) It was hoped that by omitting the phosphate constituent of Kun's buffer the catalytic method might be adapted to the determination of manganese in bone but interference by the large excesses of calcium and phosphate ions proved an insurmountable obstacle.

It appears that the potassium periodate oxidizes Mn^{++} to MnO_4^- probably by the mechanism studied by Strickland & Spicer in 1949. The MnO_4^- then oxidizes the diethylaniline to a 1:4-quinonoid compound, possibly a diquinone of the type



The manganese is then reoxidized to MnO_4^- by the periodate. Under conditions where there is excess of periodate and diethylaniline, as in the early stages of the reaction, a direct relationship between manganese concentration and rate of reaction would be expected when only a trace of the catalyst is present.

SUMMARY

1. A procedure for the determination of amounts of manganese of the order of 5 μ mg. is described. Precise adherence to the instructions is essential for the success of the method.

2. The method cannot be applied to the determination of manganese in bone, nor, without further study, to manganese in blood.

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The Manganese in Bone

BY H. FORE AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

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Manganese is generally agreed to be essential for the prevention of perosis, a bone deformity of chicks and turkeys, and it may perhaps also be essential for normal bone formation in mammals. The mode of action of manganese in calcium and phosphorus metabolism has not been established but a role as activator of phosphatase has been suggested because chicks receiving added manganese show a rise in the manganese content of bone and an increase in phosphatase activity.

The present work is concerned not only with the manganese content of bone but also with the distribution of the element among its constituent parts, in the hope of finding new evidence pertinent to the elucidation of function.

EXPERIMENTAL

Analytical methods

Emission spectrography. A Littrow type quartz spectrograph (Hilger E_1) was used in two settings covering the regions 2260–2880 and 2870–5050 Å. on 10×4 in. (25×10 cm.) plates. The light source was a d.c. arc (230 V.) between graphite or graphite-copper electrodes. For most of the work the copper-on-graphite electrodes were used; two 1 cm. lengths of pure copper rod (5 mm. diam.) were pushed into holes bored into the ends of graphite rods (9.5 mm. diam.). When the arc was running the electrodes became much hotter than simple copper electrodes and trace metals present in ash placed on the lower electrode were well volatilized into the flame. The *raies ultimes* of Mn (4030.8, 4033.1, 4034.5 Å.) are easily obscured by cyanogen emission bands when graphite electrodes are used and are only shown with the copper-on-graphite arc (5 amp., 3 min. exposure) when the Mn concentration in bone ash is between 1 and 10 p.p.m. The 2800 Å. triplet (2794.8, 2798.3 and 2801.1 Å.) was more readily recorded than the Mn II *raies ultimes* (2576.1, 2593.7 and 2605.7 Å.) and could be seen using graphite or copper on graphite electrodes at 1–10 p.p.m. Mn.

Calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) prepared from Analar Na_2HPO_4 and CaCl_2 revealed added Mn at 5 and 10 p.p.m. clearly, 2 and 4 p.p.m. with less regularity and certainty, and failed to show up 1 p.p.m.

Periodate method. The method of Skinner & Peterson (1930) was used in a modified form ensuring complete solution of the sample in H_3PO_4 .

Materials and procedures

Fresh ox femurs from the abattoir were used.

(i) After removing flesh and marrow, the bone was cut into sections which were then reduced to shavings on a lathe, taking care to avoid contamination. The shavings, consisting of both compact and cancellous bone, were subjected to extraction with glass-distilled water saturated with CHCl_3 . Three fractions were obtained, an aqueous phase, the residual shavings, and fat removed by means of ether from the extract. The bone marrow was shaken with ether for 4 hr. and on standing aqueous and ethereal phases separated, with gelatinous material at the interface. Much of this material appeared to consist of cell-wall debris from the marrow tissue, and was subsequently separated and washed with glass-distilled water. The two aqueous extracts were separately evaporated to dryness and the residues incinerated. The two ethereal extracts were washed with water, dried over Na_2SO_4 , and the ether removed from measured portions and the residues burned off. The shavings and the 'cell-wall debris' were each dried and ashed. The six samples of ash were examined spectrographically on graphite and on copper-on-graphite electrodes.

(ii) (a) Fresh bone shavings were decalcified as follows: shavings (20 g.) were shaken with 0.1N- HNO_3 (1 l.) made from redistilled acid diluted with glass-distilled water. The suspension was filtered and the filtrate and residue subjected to emission spectrum analysis.

(b) The above treatment was repeated on a fresh sample (20 g.) and the residual shavings were left to stand overnight in a further 0.5 l. of 0.1N- HNO_3 . The second extract and the (largely organic) residue were examined spectrographically. The very small amount of ash from the decalcified material was diluted with 'specpure' $(\text{NH}_4)_2\text{SO}_4$.

(iii) Two types of shavings were obtained from an ox femur, namely compact bone from the shaft and cancellous bone tissue from the head of the femur. Aqueous extracts from each type of material were made and portions were incubated with disodium phenylphosphate at pH 9 with suitable controls. After 16 hr. the solutions were tested for free phenol using 2:6-dichloroquinone chloroimide. The extracts were also tested spectrographically for Mn.

(iv) Another fresh ox femur was cut into sections and the marrow removed as completely as possible. The different portions of bone consisted of (a) predominantly epiphyseal bone from the head, (b) a mixture of epiphyseal and diaphyseal bone, and (c) diaphyseal bone from the shaft.

The separate portions of bone were incinerated and the proportions of ash determined.

(v) A portion of bone shaft was cleaned and turned on the lathe. Three experiments were carried out on the shavings. (a) 30 g. were packed in a 'chromatography' tube and covered with 0.1N-citric acid solution (made up in glass-distilled water). A dropping funnel was affixed to the top by means of a rubber bung and 3 l. of citric acid solution were allowed to percolate slowly through the shavings. Finally the contents of the tube were removed, dried and incinerated; the percolate was also dried and reduced to ash. The ash from the partly decalcified shavings was examined spectrographically and the ash from the percolate was analysed for Mn by the periodate method. (b) 16.6 g. of shavings were treated similarly and 9.3 g. of ash were obtained from the percolate and 0.31 g. from the residue in the tube. Both fractions were tested for Mn. (c) 30 g. of shavings were treated as before and the percolate collected in five fractions which were dried and incinerated separately. The residue in the tube consisted of an upper translucent portion and a lower opaque portion; the two portions were separated and each reduced to ash and examined for Mn.

RESULTS

Exp. (i) showed that the water-extracted shavings contained manganese (< 5 p.p.m.), but the ash from soluble material certainly contained no more. The aqueous extract from the bone marrow did not contain detectable amounts of manganese, but the ash from the cell-wall debris was richer than the total ash; it was enriched in manganese (10 p.p.m.) and in all other metals detected in bone (Al, B, Ba, Cu, Fe, Mg, Na, Pb, Sn, Sr and Zn) except potassium. Manganese was also detectable in the ash from both fatty fractions. The experiment showed that only a minute fraction of the total manganese in bone was present in a water-soluble form, and on the assumption that the bone phosphatase is extractable, little if any manganese can be directly attached to it. The small amount of manganese accompanying the fat may have been attached to the phosphoric acid residues of phospholipins.

Exp. (ii) showed that the organic matter remaining after decalcification with nitric acid retained a small amount of inorganic matter which was richer (on ashing) in manganese, copper, and iron than the original ash from the untreated shavings. The Mn content was about 50 p.p.m.

Exp. (iii) showed that alkaline phosphatase could be extracted by means of water from both epiphyseal and diaphyseal bone. Manganese was detectable only in the ash from the epiphyseal extract. In any case there was no sign of preferential extraction of manganese as a congener or integral part of phosphatase.

Exp. (iv) showed clearly that the percentage of ash varies for the different portions of femur (Table 1).

Table 1. *Ash contents of various parts of an ox femur*

	Wt. of bone (g.)	Wt. of ash (g.)	Percentage ash
Epiphyseal portions (near head)	118	37	31
	97	26	27
	157	51	32
	153	52	34
Diaphyseal (shaft)	23	13	57
	23	13	57
Mixed type	95	37	39
	142	54	38

Exp. (v) (a) yielded 14.4 g. of ash from the percolate and 2.8 g. of ash from the residue in the tube (i.e. 57.2% ash on the 30 g. used). The manganese content of the percolate was estimated, by a colorimetric procedure using periodate oxidation, to be 8 µg. The bone shavings were estimated to contain 15 µg. so that approximately half the manganese was retained. Similarly, in Exp. (v) (b), 16.6 g. of shavings yielded 9.3 g. of ash from the percolate and 0.31 g. from the residue, and it is estimated that approximately one-fifth of the total manganese was retained in the latter. In Exp. (v) (c) 56% of ash was obtained altogether, and within experimental error the manganese was extracted with the calcium phosphate, at first slowly and then more quickly. The proportion of the total manganese remaining in the translucent residue was quite small, but expressed in p.p.m. on the ash it was high (100 p.p.m.).

The diaphyseal bone ash contained 1.44 p.p.m. ($\pm 5\%$) of manganese and the epiphyseal bone ash 1.41 p.p.m. ($\pm 15\%$). Now the fresh diaphyseal bone contains 57% of ash so that the manganese content is about 0.51 p.p.m. The fresh epiphyseal bone contains 31% of ash corresponding with about 0.27 p.p.m. of manganese. Thus, although the manganese content of bone varies along its length, this is due entirely to the varying amount of calcium phosphate deposited. It may be concluded that most of the manganese in ox bone is deposited with the inorganic salts; but a small proportion is definitely associated with the organic matrix.

Compared with the total, the amount of manganese (if any) which is attached to water-soluble phosphatases is negligibly small.

SUMMARY

1. Spectrographic studies and determinations based on periodate oxidations show that the manganese content of ox femur is of the order 0.27–0.51 p.p.m. depending on the type of bone, compact

or cancellous. Irrespective of the type this corresponds to about 1.4 p.p.m. expressed on the ash.

2. Most of the manganese is associated with the inorganic matrix, but a small proportion is bound to the organic matrix.

3. Little manganese, if any, accompanies the water-soluble alkaline phosphatase.

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Manganese in Rabbit Tissues

By H. FORE AND R. A. MORTON

Department of Biochemistry, University of Liverpool

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Although many papers have appeared on the manganese contents of the tissues of various animals, in no case has a survey been recorded of the concentrations in all the tissues of a single animal.

Such a survey might provide a better clue to function than comparisons of the manganese contents of tissues from different individuals or species.

EXPERIMENTAL

Animals and materials

The rabbit was chosen for this investigation because it is of a convenient size for obtaining reasonable amounts of small bodies such as the pituitary and pineal glands. Four animals were used; samples of most tissues were obtained from an adult female chinchilla and of the other tissues from the remaining three. Specimens of incompletely separated adrenal cortex and medulla, thymus and thyroid were obtained from a resting adult female chinchilla; specimens of mammary gland, thyroid and lung were obtained from a lactating female albino, and samples of pancreas, thyroid, testes and spinal cord from an adult male chinchilla. Liver samples from each animal were used as controls. The pineal and pituitary glands from the last two animals were examined. The separation of the duodenal mucosa and muscular tissue was only approximate. All dissections were made with carefully cleaned stainless-steel instruments.

Using portions of kidney and liver from the first rabbit, homogenates were prepared and dialysed in cellophan bags against changing glass-distilled water for 10 days, toluene being added as preservative. The residue in each bag was divided into two portions and the Mn content of each determined.

Method of analysis

Mn was determined by a modification (Fore & Morton, 1952*a*) of a method described by Kun (1947) based on the catalytic oxidation of diethylaniline by Mn^{++} and periodate to give a coloured product absorbing at 470 $m\mu$. The experimental error is from ± 10 to $\pm 20\%$ depending on the size of the sample.

RESULTS

The findings on rabbit tissues are recorded in Table 1. The manganese contents based on fresh tissue weight are listed in decreasing order in Table 2. For comparison, (a) the values reported in the literature for each rabbit tissue have been averaged, and (b) the values reported for each type of tissue irrespective of species have been averaged. The published figures for the rabbit were obtained from papers by Bertrand & Medigreceanu (1912*a, b*; 1913), Ellis, Smith & Gates (1947), Lorenzen & Smith (1947), Lund, Drinker & Shaw (1921), Ray (1938) and Richards (1930). The figures in the right-hand column of Table 2 are averages obtained by attaching the same weight to all the published figures which can be traced. The number of papers consulted is about thirty-five and a full list of references would take up too much space.

The results of the dialyses are given in Table 3, and if the figures 2.0 and 1.2 p.p.m. are accepted for the manganese contents of the liver and kidney homogenates, respectively, it appears that in the liver 74% of the manganese, 72% of the dry matter

Table 1. *Experimental data on rabbit tissues*

Tissue	Fresh tissue wt. (g.)	Dry matter (%)	Ash as		Manganese content in p.p.m. in		
			% of fresh tissue	% of dry tissue	Fresh tissue	Dried tissue	Ash
Pituitary	0.0078	12	—	—	3.9	34	—
Pituitary	0.0071	14	—	—	1.4	9.7	—
Pituitary	0.0063	24	—	—	1.8	7.6	—
Grey hair	0.713	90	1.5	1.6	0.99	1.1	67
White hair	0.816	87	0.56	0.65	0.98	1.1	170
Hide	1.32	46	0.72	1.6	0.38	0.82	53
Fascia	4.97	23	1.1	4.7	0.024	0.10	2.2
Spleen	1.51	22	1.0	4.5	0.22	0.97	21
Bone marrow	0.640	53	0.99	1.8	0.045	0.087	4.7
Ileum	0.723	18	1.1	6.0	1.7	9.6	160
Kidney (one)	7.11	26	1.3	4.9	1.2	4.7	97
Duodenum	1.41	20	1.2	6.1	1.1	5.6	92
Mucosa	1.03	18	1.2	6.8	1.6	8.6	130
Muscularis	2.22	17	0.91	5.5	0.50	3.1	55
Salivary gland	1.42	22	1.6	7.1	1.4	6.2	88
Stomach	1.75	23	1.1	4.7	1.0	4.4	95
Caecum	1.08	17	0.79	4.7	0.82	4.9	100
Bile	1.61	20	1.7	8.5	0.48	2.4	28
Heart	1.06	22	1.0	4.5	0.28	1.3	28
Liver	3.84	30	1.6	5.3	2.0	6.7	130
Liver	3.03	29	1.5	5.3	2.2	7.6	140
Liver	2.79	24	1.2	4.9	2.0	8.4	170
Liver	3.29	25	1.2	4.9	2.2	8.7	180
Pancreas	0.225	74	1.1	1.5	1.6	2.1	140
Pancreas	0.297	26	1.5	5.8	1.6	6.2	110
Adrenals (two)	0.342	49	1.5	3.1	0.67	1.3	44
Cortex	0.290	42	1.1	2.7	0.56	1.3	50
Medulla	0.027	39	—	—	0.60	1.5	—
Ovaries (two)	0.684	23	1.5	6.3	0.60	2.6	41
Mammary gland	1.88	62	0.31	0.50	0.25	0.41	82
Mammary gland (lactating)	0.738	45	1.2	2.7	2.2	4.8	180
Brain	2.75	22	1.5	6.9	0.36	1.6	24
Muscle	2.07	23	1.2	5.0	0.13	0.56	11
Pineal	0.0061	15	—	—	3.8	26	—
Pineal	0.0018	—	—	—	0.83	3.3	—
Gall bladder	0.431	27	1.7	6.6	0.91	3.4	53
Nerve	0.178	41	1.4	3.5	0.086	0.21	6.1
Thyroid	0.0798	34	1.3	3.7	0.29	0.84	23
Thyroid	0.122	33	0.25	0.75	0.21	0.64	85
Thyroid	0.112	26	1.3	4.7	0.21	0.79	17
Testes (two)	0.0421	32	—	—	0.36	1.2	—
Spinal cord	0.393	31	1.7	5.5	0.39	1.3	23
Lung	0.868	18	0.99	5.4	0.011	0.061	1.1
Thymus	0.018	67	—	—	0.45	0.67	—

and 92 % of the ash was dialysable. In the kidney 41 % of the manganese, 49 % of the dry matter and 88 % of the ash was dialysable.

DISCUSSION

Although simple distribution studies of elements or compounds have in some instances provided valuable clues to specialized function the figures given in Table 2 do not indicate a localized role. The ubiquitous occurrence of manganese in the animal body suggests a role in general cell metabolism as opposed to metabolic processes characteristic of particular tissues. Such a role for manganese would not of course exclude other roles restricted to those tissues with a specially higher manganese content.

Dialysis of kidney and liver homogenates showed that some of the manganese is loosely held and some of it very firmly combined. If it is assumed that in all tissues having somewhat higher manganese contents, a portion of the manganese is dialysable, then the already narrow range for total manganese (Table 2) becomes even narrower for the 'bound' manganese. The small range of manganese concentrations is consistent with a general metabolic role. The fact that little or no dialysable manganese is present in ox retinas which contain only 0.13 p.p.m. of manganese (see Fore & Morton, 1952c) supports the idea that it is only those tissues relatively rich in manganese which contain the element in dialysable form.

Indications have been obtained in other work (Fore & Morton, 1952b) that some of the manganese in bone and bone marrow is attached to the cell membranes. It is therefore interesting to consider the average proportion of manganese to protein in the tissues. If 0.5 p.p.m. (Table 2) is taken as the approximate tissue content of non-dialysable

protein would need to have a molecular weight of the order 10⁶. This figure, though large, is not impossibly so since, for example, the muscle protein myosin has a molecular weight of that order. The concept of manganese attached to the cell membrane is not entirely new. Albert, Rubbo, Goldacre & Balfour (1947) suggested that oxine inhibited bacterial

Table 2. Rabbit tissues arranged in order of decreasing manganese content

Tissue	Mn content in fresh rabbit tissue (p.p.m.)	Averages of figures recorded in the literature for rabbit	Average of figures recorded in the literature for a wide range of species	Tissue	Mn content in fresh rabbit tissue (p.p.m.)	Averages of figures recorded in the literature for rabbit	Average of figures recorded in the literature for a wide range of species
Bone (long)	—	3.5	3.3	Adrenal medulla	0.60	—	—
Pituitary	2.4	—	2.5	Adrenal cortex	0.56	—	—
Pineal	2.3	—	—	Duodenal muscularis	0.50	—	—
Mammary gland (lactating)	2.2	—	0.26	Bile	0.48	—	0.90
Liver	2.1	1.9	2.5	Thymus	0.45	—	1.6
Ileum	1.7	—	—	Spinal cord	0.39	—	0.60
Duodenal mucosa	1.6	—	—	Hide	0.38	0.20	0.40
Pancreas	1.6	2.3	1.9	Intestine	—	0.38	0.45
Salivary gland (submaxillary)	1.4	—	1.1	Testes	0.36	0.40	0.50
Kidney	1.2	1.1	1.2	Brain	0.36	0.60	0.40
Duodenum	1.1	—	1.9	Uterus	—	0.35	0.33
Stomach	1.0	0.90	0.80	Heart	0.28	0.21	0.34
Colon	—	1.0	0.75	Mammary gland (resting)	0.25	—	0.14
Hair	0.99	1.2	2.4	Thyroid	0.24	—	0.55
Gall bladder	0.91	1.0	1.1	Spleen	0.22	0.55	0.40
Caecum	0.82	0.43	0.43	Muscle	0.13	0.14	0.18
Salivary gland (parotid)	—	—	0.70	Milk	—	—	0.045
Intestine (small)	—	0.70	0.45	Bone marrow	0.045	—	<0.050
Adrenals	0.67	—	0.40	Blood	—	0.03	0.040
Lymph nodes	—	—	0.65	Fascia	0.024	—	—
Ovaries	0.60	—	0.55	Lung	0.011	0.47	0.20
				Urine	—	0.00	0.004
				Parathyroid	—	—	—

Table 3. Retention of manganese after dialysis of rabbit liver and kidney homogenates

Wt. of tissue homogenate (g.)	Dialysis residue		Ash on dry matter (%)	Mn from sample (μmg.)	Mn (p.p.m.) on	
	Dry wt. (g.)	Ash wt. (mg.)			Dry wt.	Ash wt.
13.9 g. liver	0.593	8.6	1.5	4060	6.9	470
	0.583	9.6	1.6	3410	5.8	360
Totals	1.177	18.2	—	7470	—	—
Means	—	—	1.6	—	6.4	420
5.67 g. kidney	0.342	4.5	1.3	2270	6.6	510
	0.402	4.8	1.2	1750	4.4	360
Totals	0.744	9.3	—	4020	—	—
Means	—	—	1.3	—	5.5	440

manganese and 10% as an average for protein in tissue, then the proportion of manganese with respect to the protein is 5 p.p.m. There will thus be 55 parts of manganese to 11 million parts of protein and assuming one (or two) molecules of protein per atom of manganese a protein of very high molecular weight is implied. If the manganese is, however, preferentially attached to the cell-wall protein such

growth by combining with essential metals in the bacterial surface, and Gale (1949) has shown that oxine inhibits glutamic acid assimilation in bacteria by combination with a metal in the cell, probably manganese. The relatively high manganese contents of liver and lactating mammary gland could be accounted for, at least in part, by a high arginase content, and

the somewhat high manganese content in the tissues of the intestinal tract might similarly be attributed to manganese present as part of the peptidase system.

SUMMARY

1. The manganese contents of rabbit tissues have been determined, and the results compared with those obtained by previous workers.
2. The range covered by the results is narrow,

especially as some of the tissues relatively rich in manganese contain much of it in dialysable form.

3. It is suggested that manganese participates in general cell metabolism while attached to the cell-wall protein.

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Manganese in Eye Tissues

By H. FORE AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

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As part of a programme of research on the biochemistry of vision the manganese contents of the eye tissues of the ox have been determined. The only similar study appears to be that of Tauber & Krause (1943), who used an extension of the periodate method (Wiese & Johnson, 1938) on samples obtained by taking tissues from the eyes of a large number of oxen. The method used here is the modified catalytic method (Fore & Morton, 1952a); it is sufficiently sensitive to be applicable to the tissues from single eyes.

EXPERIMENTAL

Left and right eyes from each of two oxen were studied separately. After removing muscle, etc., attached to the sclera, each eye was dissected using carefully cleaned stainless-steel instruments. Separation of the tissues was complete except perhaps for the conjunctiva, and minute fragments of iris tissue were sometimes present in the sample of vitreous humour. The optic nerve was cut out of the sclera, leaving no nerve tissue in the sclera sample, from which choroid pigment was carefully removed. The cornea, lens, sclera, optic nerve and conjunctiva were washed with glass-distilled water and touched on filter paper (Whatman no. 50) before weighing. The retina, choroid and the vitreous and aqueous humours were placed directly into crucibles.

The iris, which adheres to the delicate membrane retaining the vitreous humour, was touched on filter paper to remove traces of vitreous humour.

In a later experiment a retinal homogenate was prepared from about forty ox retinas, toluene added and the brei dialysed against changing glass-distilled water. The Mn contents of samples removed from the cellophan bag at 70 and 180 hr. were determined by the modified catalytic method.

RESULTS

The results of the manganese determinations of the eye tissues are given in Tables 1 and 2. In Tables 3-5 the tissues are arranged in order of decreasing manganese content alongside the values obtained by Tauber & Krause, who did not examine the aqueous humour nor the contents on an ash-weight basis.

The results of the dialysis experiment are given in Table 6. The weight of the homogenate used for dialysis expressed as dry weight (*) was calculated using the percentage dry weight (not given) obtained for two samples of the homogenate. Then, from the manganese content of these samples and the calculated dry weight, the absolute amount of manganese in the homogenate used for the dialysis (†) was obtained.

Table 1. *Experimental data on ox eye tissues*

Tissue	First experiment				Second experiment			
	Wt. of fresh tissue (g.)	Dry matter (%)	Ash on fresh tissue (%)	Mn from tissue (μmg.)	Wt. of fresh tissue (g.)	Dry matter (%)	Ash on fresh tissue (%)	Mn from tissue (μmg.)
Conjunctiva	1.10*	20	0.39	286	0.169	19	1.0	21.0
	0.601*	30	0.55	164	0.085	19	0.83	19.6
Cornea	0.723	18	0.57	33.4	0.666	22	0.95	26.3
	0.788	18	0.72	Lost	0.644	23	0.89	27.8
Aqueous humour	1.25	1.3	0.85	12.8	1.62	1.2	0.94	21.8
	0.937	1.3	0.86	14.3	1.63	1.2	0.88	15.7
Iris	0.678	16	0.77	71.0	0.860	18	0.92	113
	0.821	14	0.93	104	0.838	18	1.2	91.0
Lens	2.54	34	0.68	31.6	2.41	35	0.70	16.7
	2.39	40	0.73	29.1	2.36	36	0.71	24.8
Vitreous humour	13.8	1.2	0.86	110	16.8	1.1	0.81	83.5
	12.2	1.3	0.87	163	16.2	1.1	0.82	104
Retina	0.624	11	0.87	112	0.760	12	0.90	76.8
	0.656	11	0.93	91.5	0.715	13	1.0	71.7
Choroid	0.514	18	1.07	69.1	0.480	21	1.3	49.8
	0.429	18	1.21	57.8	0.445	20	1.4	45.3
Sclera	5.15	27	0.48	675	5.58	30	0.43	1010
	5.00	29	0.54	1130	5.42	30	0.53	920
Optic nerve	0.463	28	1.06	36.8	0.639	28	1.2	9.0
	0.399	32	1.15	39.8	0.962	27	1.2	12.9

* Possibly contaminated with sclera.

Table 2. *Manganese and ash contents of ox eye tissues determined in the present work and compared with the findings of Tauber & Krause (1943)*

(Figures in brackets are the extreme values for individual determinations.)

Tissue	Ash on fresh tissue (mean) (%)	Manganese (p.p.m.) of				Ash
		Fresh tissue		Dried tissue		
		Present work	Tauber & Krause	Present work	Tauber & Krause	
Conjunctiva	0.70	0.23 (0.13-0.27)	0.10 (0.069-0.11)	1.0 (0.68-1.3)	0.28 (0.23-0.37)	39 (12-67)
Cornea	0.80	0.044 (0.04-0.046)	0.11 (0.094-0.13)	0.24 (0.18-0.29)	0.44 (0.37-0.49)	6.4 (4.2-8.2)
Aqueous humour	0.89	0.012 (0.010-0.015)	—	0.98 (0.78-1.2)	—	1.5 (1.1-1.8)
Iris	0.93	0.12 (0.11-0.13)	0.24 (0.21-0.29)	0.73 (0.62-0.89)	0.92 (0.79-1.2)	13 (9-14)
Lens	0.71	0.010 (0.007-0.012)	0.18 (0.14-0.22)	0.031 (0.02-0.037)	0.52 (0.41-0.63)	1.5 (1-1.8)
Vitreous humour	0.85	0.008 (0.005-0.013)	0.031 (0.019-0.042)	0.69 (0.44-1.1)	1.5 (0.93-2.1)	0.95 (0.6-1.5)
Retina	0.93	0.13 (0.10-0.18)	0.32 (0.24-0.39)	1.1 (0.8-1.6)	2.6 (2.0-3.2)	15 (10-21)
Choroid	1.2	0.12 (0.10-0.14)	0.21 (0.17-0.32)	0.63 (0.50-0.76)	0.71 (0.59-1.1)	9.9 (5.6-13)
Sclera	0.50	0.18 (0.13-0.23)	0.09 (0.078-0.13)	0.61 (0.48-0.77)	0.32 (0.25-0.42)	36 (27-43)
Optic nerve	1.1	0.052 (0.013-0.10)	0.26 (0.22-0.31)	0.18 (0.049-0.31)	1.0 (0.83-1.2)	4.7 (1.1-8.7)

